THE PHYSIOLOGICAL EFFECTS OF IONOPHORETIC CROWN ETHER ON *ESCHERICHIA COLI*

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ABSTRACT

Crown ether, being the first synthesized organic compound with high complexability towards alkali metal ions, has been widely used in the industrial chemistry and in biochemical studies. It is toxic to animal. In this investigation, the 15-crown-5 and the 18-crown-6 series are studied with respect to their physiological effect on bacterial system.

It is shown by solvent extraction method that 15-crown-5 is more selective towards sodium ion whereas for all 18-crown-6 compounds, potassium is the preferred ion. In the bacterial system, these compounds are toxic at high concentrations, with a minimal inhibitory concentration (MIC) at about 5×10^{-2} M. In general, they reduce bacterial respiration slightly at low crown ether concentrations but generate rather significant inhibition at concentration above 10^{-4} M. This toxic effect is also shown in the bacterial growth curve, with the presence of a lag, a slight decreased logarithmic growth rate and a lower stationary phase population. The toxicity of the parent 18-crown-6 is unique among all the members of 18-crown-6 family studied : it is the only crown ether that has its

toxicity reducible by the addition of a sublethal dosage of potassium ion. This phenomenon is a common feature among all potassium selective ionophores, such as valinomycin and monactin.

At sublethal level of 15-crown-5 or 18-crown-6 alone, E. coli grows into filamentous cell. These filamentous cells are motile, lack of septum, polynucleated, and capable of reversing to normal short cells by reculturing in the absence of the crown ether. The colony morphology of these filamentous cells on agar plate also exhibits abnormal irregular pattern. Again, addition of potassium alone in the growth medium results in upshifting the 18-crown-6 effective concentration for bacterial filamentous cell formation. The whole cell protein pattern of these filamentous cells indicates the absence of three major protein bands in the SDS polyacrylamide gel electrophoresis.

An ion transport study with rubidium-86 indicates 18-crown-6 is effective in enhancing the permeability of the alkali metal ion, with rubidium mimicing potassium, through the bacterial cell membrane. With all these observations taken together, it is suggestive that the effect of crown ether in the bacterial system

in altering the growth kinetics as well as in the change of the cell morphology may have stemmed mainly from its ionophoretic effect.

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I. INTRODUCTION

1. Crown Ether

A Brief History of the Study of Crown Ether Chemistry

In spite of the fact that crown ether was first reported in 1937 (Luttringhaus and Ziegler, 1937), systematic investigation of its synthesis, complexation capabilities and potential applications in industry have only been started after Pedersen's pioneer publication in 1967 (Pedersen, 1967) . Crown ether, as a family, is characterized of having different number of $-OCH_2CH_2-$ constituting units either substituted or unsubstituted, joined covalently in a macrocyclic ring. The number of repeating units normally varies from three to ten, of which, only those containing five or six oxygen atoms in the ring have attracted the widest attention. These crown ethers include 1,4,7,10,13-pentaoxacyclopentadecane and 1,4,7,10,13,16-hexaoxacyclooctadecane, which have been abbreviated as 15-crown-5 and 18-crown-6 correspondingly according to Pedersen's nomenclature system (Pedersen, 1967). The ethylene unit of crown ether can be further substituted giving rise to a whole family of the monocyclic crown ether. Dicyclohexyl-18-crown-6, benzo-18-crown-6 and 4'-methyl-benzo-18-crown-6 are the three 18-crown-6

compounds among the most widely used (Fig. 1).

Application of Crown Ether

Crown ether can form stable complexes with alkali and alkaline earth (Pedersen, 1967 & 1970), transition and lanthanide metal ions (Christensen et al., 1971; Pedersen & Frendorff, 1972; Cassol et al., 1973). The molecule has in general a central hydrophilic cavity with a hydrophobic external ring. Chemically the central oxygen helps to stabilize the salt in organic solvent by the formation of ion pair to enable reaction to proceed in the presence of the cation (Alper et al., 1977; Sam & Simmons, 1972; Wong, 1978). Because of this unique property, the crown ether can facilitate many ionic reactions to be carried out in systems containing nonaqueous solvents.

The complexation capability of crown ether depends on the ring size of the crown ether and is highly ion specific. Because of its ion selectivity, crown ether is extensively used in selective ion electrode, as selective agent and as ion chelating resin (Kimura et al., 1979; Ryba & Petranck, 1973).

In biochemistry, the ion transport property of this macrocyclic molecule enab e it to be employed as

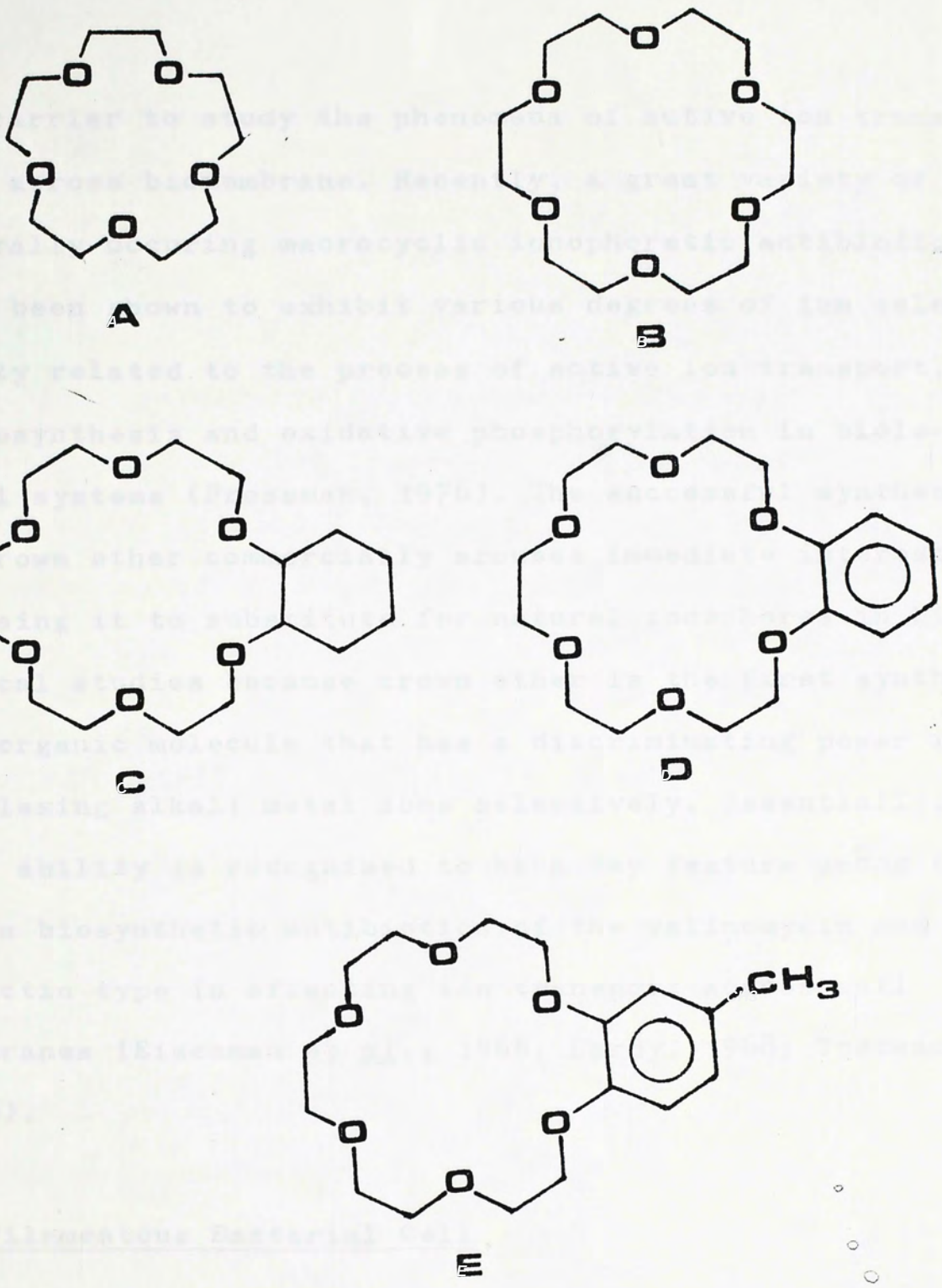


Figure 1. Structure of Crown Ethers.

(A) 15-crown-5, (B) 18-crown-6, (C) dicyclohexyl-18-crown-6, (D) benzo-18-crown-6, and (E) 4'-methyl-benzo-18-crown-6.

ion carrier to study the phenomena of active ion transport across biomembrane. Recently, a great variety of naturally occuring macrocyclic ionophoretic antibiotics have been shown to exhibit various degrees of ion selectivity related to the process of active ion transport, photosynthesis and oxidative phosphorylation in biological systems (Pressman, 1976). The successful synthesis of crown ether commercially arouses immediate interest in using it to substitute for natural ionophores in biological studies because crown ether is the first synthetic organic molecule that has a discriminating power in complexing alkali metal ions selectively. Essentially, this ability is recognized to be a key feature among the known biosynthetic antibiotics of the valinomycin and monactin type in affecting ion transport across cell membranes (Eisenman et al., 1968; Lardy, 1968; Tosteson, 1968).

2. Filamentous Bacterial Cell

Filamentous Bacterial Cell Formation

It is recognized that several types of bacteria or their mutants can be converted into filamentous form by a variety of environmental factors, such as the presence of antibiotics and other metabolic inhibitors, the

deficiency of essential growth factors, and the change in the pressure, temperature or other physical treaments such as radiation. A list of these factors is included in Tables 1 & 2.

Although defects in the mechanism of cell division have been demonstrated to result in filamentous cell formation, no detailed biochemical study towards the understanding of this phenomenon has been thoroughly done.

Normal and Abnormal Bacterial Cell Division

Bacterial cell division is a series of cellular events including cell growth, nuclear synthesis, nuclear division and cytokinesis (cross-septation). For the newly grown bacterial cell of the binary fission type to reach a physiological state identical to that of its parent, it involves a replication of the genetical material, a segregation of the two equal sets of genes, an extension of the cell wall and its membranous confinement, a doubling of the total cell mass, a compartmentalization of the cellular components and a total separation of the two individual cells. It is obvious that disturbance in any of the above mentioned steps will lead to unsuccessful cell d[illegible]ion which may end up in

Table 1. External Factors that Generate Filamentous Cell Formation

Factor	Bacterium	Reference
Elevated pressure	*Escherichia*	ZoBell & Cobet, 1964
	Serratia	ZoBell & Oppenheimer, 1950
Elevated temperature	*Escherichia*	Hoffman & Frank, 1963
		Hirota *et al.*, 1970
		Kamiryn & Strominger, 1974
		Maclean & Munson, 1961
		Ron & Davis, 1971
	Bacillus	Bulman & Stretton, 1975
		Chaloupka *et al.*, 1974
		Ferroni & Inniss, 1973
		Hitchins & Sadoff, 1974
	Clostridium	Barker & Beck, 1942
		Terry *et al.*, 1966
Radiation	*Escherichia*	Adler & Hardigree, 1964a,b & 1965a,b
		Flanders, *et al.*, 1964
		Otsuji *et al.*, 1974
		Payne, *et al.*, 1956
		Witkin, 1947
		Lea *et al.*, 1937
		Spoerl *et al.*, 1954
Mg deficiency		Webb, 1949 & 1951
Unfavorable pH		Wahlin & Almaden, 1939
Nutrient deficiency	*Lactobacillus*	Glick *et al.*, 1960
Metal ion deficiency	*Clostridium*	Shankar & Bard, 1952
Nucleic acid starvation	*Thermobacterium*	Jeener & Jeener, 1952
Thymine deficiency	*Escherichia*	Cohen & Barner, 1954
Vitamin B_{12} deficiency	*Lactobacillus*	Beck *et al.*, 1962
		Kitahara & Kusaka, 1961

Table 2. Formation of Filamentous Cell by the Addition of Various Chemicals

Type of Chemical	Chemical	Bacterium	Reference
DNA synthesis inhibitor	Nalidixic acid	Escherichia	Goss et al., 1964
	Mitomycin C	Escherichia	Reich et al., 1961
	Quindoxin	Escherichia	Suter et al., 1978
DNA damaging agent	Dihydroxymethyl furatrizine	Escherichia	Iida & Koike, 1977
	Nitrogen & sulfur mustard	Escherichia	Harold & Ziporin, 1958 Bryson, 1948
Thymine analogue	5-Bromouracil	Escherichia	Zamenhof et al., 1956
Nucleic acid analogue	5-Flurorouracil deoxyriboside	Escherichia	Cohen et at., 1958
Anti-tumor drug	Cis-platinum (II)-diaminodichloride	Escherichia	Thomson et al., 1972
Antibiotics	Cinoxacin	Escherichia	Mardh & Arhammer, 1978
Cell wall synthesis inhibitor	Penicillin	Escherichia	Strominger et al., 1971
Surface active agent	Sodium dodecyl sulfate	Escherichia	Onitsuka et al., 1975 & 1979
		Bacillus	Mendelson et al., 1976
Others	Azaserine	Escherichia	Maxwell & Nickel, 1954
	β-Lactam	Escherichia	Spratt, 1975
	D-Amino acid	Erwinia	Grula, 1960
	3-Chloropropane-1,2-diol	Escherichia	Bulman & Stretton, 1974
	Phenethyl alcohol	Escherichia	Berrah & Konetzka, 1962

filamentous cell formation.

Since cell division occurs at a fixed time after the termination of each round of DNA replication (Donachie, 1969; Pritchard et al., 1969). An intimate relation between these two processes is expected. More recently, it is shown that the termination of a round of DNA replication triggers the bacterial cell to divide (Clarke, 1968; Cooper & Helmstetter, 1968). When DNA synthesis is stopped in exponentially growing cultures, only those cells that have completed a round of DNA replication possess the ability to divide (Helmstetter & Pierucci, 1968). On the other hand, if the bacterium is subjected to conditions, thought to have interfered with its DNA synthesis (e.g. the presence of thymidine analogues, irradiation or thymine starvation), a filamentous cell type is formed, presumably because cell septation is inhibited.

Bacteria are prokaryotic organisms, devoid of a morphologically individualized nucleus and of a mitotic apparatus which in eukaryotes handles the equipartition of the chromosomes. Some cytological studies provide evidences that DNA molecules are linked to the cell membrane in bacterium (Ellar et al., 1967; Fuhs, 1965 & 1969; Iterson, 1961 & 1965; Ryter, 1968). The growth of

the bacterial membrane may have played a role analogous to that of the mitotic spindle in the eukaryotic cells. It has been proposed in a model that the bacterial cell membrane grows only in a central zone, which is located between the attachment point of two sister chromosomes (Jacob et al., 1963). Cell division occuring at the centre of the zone results in the segregation of two chromosomes into the two sister cells. However, at present, a direct observation of the site of bacterial membrane growth is lacking. It is known that the junction between DNA and the membraneous fraction is usually located at an infolding structure known as the mesosome. It is believed that at this junction, the rate of membrane growth occurs faster than that of the cell wall extension resulting in a formation of mesosome (Bazill, 1967; Rogers, 1970). Mesosomes, in a dividing cell, are generally located at the septum formation region and have been considered to be involved in septation (Chapman & Hillier, 1953; Edwards & Stevens, 1963; Ellar et al., 1967).

Furthermore, the components of the cell wall layer are synthesized at the level of the cytoplasmic membrane. The enzymes which produce cell wall components such as those of peptidoglycan (Anderson et al., 1972; Rogers; 1970), lipopolysaccharide (Hinckley et al., 1972;

Osborn et al., 1972) and phospholipid (Bell et al., 1971) are all found in the cytoplasmic membrane. It is reasonable to believe that an interaction with the cytoplasmic membrane may also affect the function of these enzyme systems which lead to a malfunction in the cell division process. This is essentially a possible reason given to explain why membrane active chemicals, such as sodium dodecyl sulfate, have been reported to cause filamentous cell formation in bacteria (Mandelson et al., 1976; Onitsuka et al., 1975 & 1979).

An essential structural component of the wall of all bacteria, except for the extreme halophiles, is the mucopeptide layer. This layer is thick in gram-positive bacteria and thin in gram-negative bacteria. The function of the mucopeptide layer is directly related to the maintenance of the cell shape, by protecting the membrane, and by forming a septum between individual cells whereas the plasma membrane alone has low strength and rigidity (Iterson & Kamp, 1969; Rogers et al., 1967). This mucopeptide consists of alternating residues of the two amino sugars, N-acetylglucosamine and N-acetylmuramic acid, joined together by 1→4β glycosidic linkages. Its biosynthesis involves the nucleoside diphosphate activated N-acetylmuramyl peptidic compounds and uridine diphospho-N-acetylglucosamine (Ghuysen et al., 1968; Osborn, 1969;

Rogers & Perkins, 1968). Both precursors are made by soluble enzymes in the cytoplasm and are transfered by membrane-bound enzymes to form a disaccharide peptide. Addition of the amino acids onto the disaccharide are achieved by stepwise transfer from the transfer ribonucleic acid (t-RNA) (Thorndike & Park, 1969). The final cross-linking reaction is supposed to be undertaken by an acceptor and a transpeptidase outside the membrane (Rogers, 1965). Evidences supported a view that the cell wall biosynthesis is localized in the region of the dividing septum (Cole, 1965; Cole & Hahn, 1962). However, these biosynthesis sites will redistribute when protein synthesis is stopped (Higgins & Shockman, 1967). The mucopeptide-hydrolyzing enzymes, consequently, are located in the septum and nascent wall synthesized region. (Shockman *et al.*, 1967). These enzymes are suggested to have several major functions : to open the 'sacculus' of mucopeptide and provide new acceptor sites for insertion of additional materials (Schwarz *et al.*, 1969; Shockman, 1965), to rearrange the existing mucopeptide for septum formation (Rogers, 1965) and to separate the compartmentalized cells(Rogers, 1970). On the same token, cell septation being a biochemical event, can also be inhibited by mucopeptide synthesis inhibitors. For example, low concentration of penicillin that causes

approximately 20 to 70 % inhibition of cell wall synthesis induces filamentous bacterial cells (Nathanson & Strominger, 1961). Other factors known to inhibit cell wall synthesis such as addition of D-amino acid, or magnesium deficiency, also cause filamentation (Grula, 1960; Garrett, 1969).

It is clear at this point as mentioned in previous paragraph, that any inhibition in one process within that series of reactions needed for cell division will lead to the production of filamentous cell.

3. Aim of the Study

Crown ether has been recently studied in animal systems and shown to have a toxic effect (Leong et al., 1974; Pedersen, 1967 & 1972; Takayama et al., 1977). On view of the widespread application of crown ether in industrial chemistry as well as studies in biological functions of the membrane, a problem of possible hazardous effect to the public health should not be overlooked. It is the aim of this study to initiate a thorough research with some of the popularly used crown ethers on a comparatively simple bacterial system to find out in more details the nature of its toxicity and the possible mech ism of s toxicity.

II. MATERIALS AND METHODS

MATERIALS

1. Chemicals

Polyethers : High purity (98 %) 15-crown-5 (1,4,7,10,13-pentaoxacyclopentadecane) and 18-crown-6 (1,4,7,10,13,16-hexaoxacyclooctadecane) were purchased from Sigma and used without further purification. Dicyclohexyl-18-crown-6 was purchased from Aldrich Chemical Company and was purified by column chromatography on activated neutral alumina with n-heptane as eluent. 4'-methyl-benzo-18-crown-6 and benzo-18-crown-6, a gift from Dr. K.H. Wong, were synthesized according to the procedures described by Pedersen (1967) and Smid et al.(1971). Polyethylene glycol 200 (molecular weight range from 190-210) and polyethylene glycol 400 (molecular weight range 390-410), purchased from British Drug House (BDH) were used without further purification. The polyether solution was usually prepared as a highly concentrated aqueous solution, depending on its solubility, and sterilized by filtering through sterile membrane filter (Millipore, pore size 0.45 μm).

Rubidium-86 : It was purchased from the Radiochemical Centre, Amersham, was in rubidium chloride form.

Alkali Metal Salts : RbCl, LiCl, KCl and NaCl were obtained from Merck and CsCl was from BDH. All these salts are of analytical grade.

2. Growth Media

Tryptone Broth (TB) : 1 % (w/v) Difco tryptone in glass distilled water was sterilized by autoclaving at 121°C for 25 minutes.

Sodium Tryptone Broth (NaTB) : 1 % (w/v) Difco tryptone and 0.5 % (w/v) NaCl in glass-distilled water was sterilized by autoclaving.

Defined Potassium Phosphate Minimal Medium (KMM) : In 500 ml of distilled water, 11.2 g K_2HPO_4, 4.8 g KH_2PO_4, 2 g $(NH_4)_2SO_4$, 0.25 g $MgSO_4 \cdot 7H_2O$, 150 mg each of L-histidine, L-threonine and L-leucine were added to make the mineral salt solution. The final pH of the medium was 7. The Carbon source of the medium was prepared by adding 10 g of gluse in 500 ml distilled water. These two media were autoclaved separately and mixed together after sterilization.

Defined Sodium Phosphate Minimal Medium (NaMM) : 1 litre of NaMM medium contained 23.1 g $Na_2HPO_4 \cdot 12H_2O$, 5.5 g $NaH_2PO_4 \cdot 2H_2O$, 2 g $(NH_4)_2SO_4$, 0.25 g $MgSO_4 \cdot 7H_2O$, 150 mg

each of L-histidine, L-threonine and L-leucine and 10 g of glucose. The medium was sterilized as mentioned above with the carbon source, glucose, autoclaved separately. The final pH was 7.

Defined Ammonium Phosphate Minimal Medium : 1 litre of 0.1 M ammonium phosphate (pH 7) medium was prepared by adjusting accurately the phosphoric acid with NH_4OH to pH 7. The medium contained in addition 2 g $(NH_4)_2SO_4$, 0.25 g $MgSO_4 \cdot 7H_2O$, 150 mg each of L-histidine, L-threonine and L-leucine; and 10 g glucose. Glucose was autoclaved separately.

Halobacterium Growth Medium : The medium was consisted of 7.5 g casamino acid (Difco), 10 g yeast extract (Difco), 3 g sodium citrate, 2 g KCl, 20 g $MgCl_2$, 20 g $MgSO_4 \cdot 7H_2O$, 250 g NaCl and 23 mg $FeCl_2$, all dissolved in 1 litre of glass-distilled water (pH 6.2).

Tetrahymena Growth Medium : It was consisted of 100 part of medium A and 1 part of medium B, both were prepared and autoclaved separately. Medium A contained 2 % (w/v) proteose peptone (Difco), 0.1 % (w/v) yeast extract (Difco) and 0.5 % (w/v) glucose. Medium B was prepared by dissolving 1 g KOH and 3.35 g $Na_2EDTA \cdot 2H_2O$ in 100 ml of glass-distilled water and the solution was adjusted to pH 5 with either HCl or KOH before further

addition of 2.5 g $FeSO_4 \cdot 7H_2O$. This final solution was then readjusted to pH 5.5. The final volume was brought up by adding glass-distilled water to a final of 1 litre. Medium B was stored in brown bottle at 0-4°C.

3. Microorganisms

Bacterial Strains : Escherichia coli K12, AW405, is gal^{-1}, gal^{-2}, thr, leu, his^{-4}, thi, lac, xyl, ara, str-r, ton, A-r, tsr-r. Salmonella typhimurium LT_2 was obtained from Prof. B.N. Ames. Streptococcus, Staphylococcus coagulate +, Staphylococcus coagulate -, Corynebacterium and Bacillus were obtained from the Agriculture and Fisheries Department of Hong Kong. Halobacterium cutiritium was obtained from Prof. C.F. Wong.

Protozoa : Tetrahymena thermophila was obtained from Dr. Stephen Ng.

METHODS

1. Determination of Extinction Coefficient (ε) of Crown Ether-Potassium Picrate Complexes

An accurately weighed potassium picrate was dissolved in a chloroform solution of crown ether, in which crown ether was slightly in excess. On complete solvation, which usually took 2-3 days at room temperature, the absorption spectrum of the crown ether-potassium picrate was determined spectrophotometrically with a Beckman spectophotometer (Model 25), and the extinction coefficient (ε) of the complex was calculated according to the Beer's law, $A = \varepsilon dc$, where A is the absorbance of the complex at the corresponding absorption maximum (λ_{max}), c is the molar concentration of the complex and d is the light path in cm.

2. Determination of Complexation between Crown Ethers and Alkali Metal Ions by the Solvent Extraction Method

Essentially 4 ml of water saturated chloroform solution of crown ether and 4 ml of chloroform saturated aqueous solution of picrate acid and an alkali metal chloride were mixed in a test tube, as modified from the method of Ng (1979) and Wong et al. (1974). The binary system was thoroughly mixed to ensure an equi-

librium was reached. This equilibrated system was left undisturbed at room temperature overnight to allow complete separation of the two phases. The chloroform layer which contained the crown ether-picrate salt complex was then collected and the absorption spectrum (300 nm — 500 nm) was determined according with a Jasco spectrophotometer (Model ORD/UV-5).

3. Determination of the Susceptibility of Microorganisms to Crown Ether Toxicity by the Test Tube Serial Dilution Method

A serial two-fold dilution of crown ether in the appropriate growth medium was prepared according to the susceptibility test described in the method of Bailey & Scott (1970). Except Halobacterium cutiritium and Tetrahymena thermophila, which had their corresponding growth medium as described in Materials, all other microbials employed tryptone broth for growth. These crown ether serially supplemented growth media with their controls were then inoculated with aliquotes of logarithmic phase freshly cultured microorganisms and incubated in a gyrotory shaking water bath (New Brunswick Scientific, Model G76). The protozoa was grown at 28°C. For microorganisms with slow growth rate, such as

Tetrahymena and Halobacterium, a longer period of approximately 40 hours was needed for significant turbidity to be developed. In each set of determination, the chemical composition of the growth medium, the incubation period as well as the incubation temperature were maintained constant so as to see only the toxic effect of the tested chemical, crown ether(Fink, 1962). The turbidity of the culture media was measured at wavelength 590 nm, with a photometer (Bausch & Lomb, Spectronic-70), using a cuvette of 5 mm light path. The lowest chemical concentration in the series that shows no turbidity increment was taken as the minimal inhibitory concentration (MIC) as described by Bailey and Scott (1970). Similarly, the LD_{50} value was determined at a chemical concentration that allowed only fifty percent growth.

4. Microscopic Observation of Bacterial Morphology

The effect of a chemical on the morphology of E. coli in a non-growing condition was observed microscopically with the phase-contrast optics (Nikon, Biophot). The bacterial cells used were prewashed three times in a buffered medium containing only 10^{-2}M phosphate at pH 7. No protein synthesis inhibitor was

added. To study the chemical effect on bacterial growth, however, a suspension of bacteria was withdrawn from a growing culture at mid-logarithmic phase instead. In all these studies, the bacteria thus employed had been grown for more than 5 generations in a medium containing the chemical of interest.

5. Determination of Bacterial Viability

Autoclaved tryptone agar (Difco, 1.5 % w/v) was cooled to 60°C in a warm water bath and a calculated amount of the crown ethers was added and mixed thoroughly before pouring plate. These plates were usually mildly desiccated overnight in 45°C incubator before use. To the chemical supplemented agar plate, an overlay tryptone agar (0.75 % w/v) containing freshly grown bacteria was added. The number of bacteria to be plated was adjusted by dilutions so that only colony-countings from 100 to 400 were used. The plates were incubated at 37°C overnight for bacterial growth and standard bacterial colony counting procedures were employed.

6. Bacterial Growth Curve Measurement

Ten ml of culture medium containing various known concentrations of the chemicals to be studied was asep-

tically dispersed into a series of optically matched, screw-capped flasks with side-arm test tube attachment. The diameter of the test tube is 12 mm. To each flask, approximately 0.3 ml aliquote of healthily grown, logarithmic phase E. coli culture was added. Usually, the initial turbidity read below 0.02 in absorbance. All flasks were incubated at 37°C in a gyrotory water bath shaker. The turbidity was measured at 590 nm at various time intervals using Spectronic-70 colorimeter, calibrated against a blank containing only the culture medium.

The lag time of the bacterial growth curve was determined according to the method of Lodge and Hinshelwood (1943). This was done by extrapolating the linear portion of the exponential phase of the growth curve to a point on the turbidity scale where the initial biomass was located. The time corresponding to this point was the lag time in the medium measured.

Growth yield was defined as the mass of the bacteria generated versus the mass of the nutrient substrate used according to Dawes & Sutherland (1976); and Stanier (1970). When none of the parameters such as growing condition, bacterial strain and nutrien medium used were altered, a comparison of the effect of chemicals on the growth yield can be obtained simply by measuring their turbi-

dity at the stationary phase correspondingly.

7. Measurement of ^{86}Rb Uptake

E. coli was cultured in defined sodium phosphate minimal medium (NaMM), containing 10 mM RbCl in place of potassium. These freshly grown logarithmic phase bacterial cells were harvested, washed twice with and resuspended in 25 mM sodium maleate buffer (pH 7). Portions of the bacterial cells were added into medium containing 25 mM sodium maleate (pH 7), 10 mM $^{86}RbCl$, and 10^{-2}M 18-crown-6. In some suspensions, 0.1 M glucose was also added as energy source for bacterial metabolism. All samples were incubated at 37°C water bath. At various time intervals, 1-ml bacterial cells were collected through millipore filter (Millipore, pore size 0.45 μm), washed with 5 ml 2 mM magnesium chloride, once with 1 ml (Harold & Baarde, 1967 & 1968).

8. Effect of 18-Crown-6 on the ^{86}Rb Efflux across *E. coli* Cell Membrane

E. coli adapted for over 20 generations in a sodium phosphate minimal medium (NaMM) with 10 mM RbCl, were transfered to a medium containing the same chemical composition, except hot ^{86}Rb was added, and was cultured

overnight at 37°C in a gyrotory shaking water bath. The bacterial cells were harvested by centrifugation at 10,000 g for 10 minutes. These cells were then washed twice and resuspended either in water or in 25 mM sodium maleate buffer (pH 7) supplemented with 10 mM RbCl. Amount of bacterial cell protein which was used as a measurement of the bacterial amount, was determined by the modified Lowry method (Markwell et al., 1978). To portions of this suspension were added different concentrations of the test chemical 18-crown-6 and incubated at 37°C in a water bath. 1-ml samples were withdrawn at appropriate time intervals and filtered rapidly through a filter membrane (Millipore, pore size 0.45 μm). It was either washed with 10 ml 20 mM RbCl for those cells previously suspended in water according to Harold & Baarda (1967 & 1968), added at ten 1-ml portions; or washed with 5 ml glass-distilled water for those cells suspended in maleate buffer (Harold et al., 1967; Veerkamp, 1978), added at five 1-ml portions. The radioactivity retained in the bacteria was determined by immersing the filters in scintillation fluid and counted with a liquid scintillation counter system (Beckman, Model LS-330). The selected counting channel was adjusted to be located at the optimum range of the ^{86}Rb energy spectrum.

9. Measurement of Bacterial Respiration

E. coli grown to logarithmic phase in a tryptone broth (TB) was harvested by centrifugation at 10,000 g for 10 minutes. The pellet was washed twice and resuspended in a phosphate buffer (pH 7.5) containing 10^{-2} M potassium phosphate, 10^{-4} M EDTA and 10^{-2} M D-glucose. Bacterial respiration was measured polarographically with a Clark oxygen electrode (Yellow Springs Instrument Co., Model 53) at 37°C in the phosphate buffer (pH 7.5). Original volume of bacterial suspension was 4 ml, addition of chemicals in aqueous solution was adjusted not to exceed 10 % (v/v) of the original suspension volume. The dilution effect after each additional volume of chemical solution was considered in calculation. The amount of bacterial cells in the suspension was determined by conventional pouring plates and counting the number of colonies development overnight. Usually the amount of bacteria used in each measurement was around 10^{12} cells, with a respiration rate approximately 0.25 $\mu l O_2$/min.

10. Microscopic Observation of Bacterial Morphology in Tryptone Broth Culture Medium Supplemented with 18-Crown-6

A serial 2-fold dilution of 18-crown-6, with the highest sublethal concentration to begin with, in 1 %

tryptone broth (TB) growth medium were prepared. For Halobacterium, the corresponding medium as described in Materials was used. With the inoculum incubated overnight in a gyrotory shaking water bath at 37°C, aliquotes of the culture were examined microscopically with phase-contrast optics (Nickon, Biophot). The concentration of the 18-crown-6 in which an alteration of bacterial morphology was noted by comparison with the bacteria grown in plain growth medium. The concentration range being studied was 10^{-4} M to 0.1 M for the 18-crown-6 family and 10^{-3} M to 1 M for 15-crown-5. In order to find the effective concentration range in which a morphological change can be introduced, the threshold and the upper concentration were determined.

11. Histochemical Studies of Bacterial Cell

To show the gross morphology of the bacterial whole cell, the Loeffler's methylene blue staining method was employed (Norris & Swain, 1971). 0.3 g methylene blue (Sigma) was dissolved in 30 ml ethyl alcohol and 100 ml distilled water. Bacterial smears were prepared by spreading aliquotes of the culture medium on microscopic slides and fixed by drying in the air. The fixed smears were allowed to be covered by the methylene blue staining

solution for 1 minute. The excess stain was then removed by washing with water and dried with a blotting paper. Bacterial cells were stained dark blue in colour.

To stain the bacterial capsule, the Hiss's capsule stain was employed (Norris & Swain, 1971). The bacterial smears were prepared as described above and was stained with 1 % aqueous crystal violet for 2 minutes followed by subsequent washing with a solution of 20 % copper sulfate. The treated specimen showed a pale blue capsule from the dark purple cell.

To locate the bacterial chromatin material, the Robinow's nuclear stain was followed with slight modifications (Robinow, 1944). The bacterial cells were harvested and chemically fixed by suspending in 3 % glutaraldehyde for 30 minutes followed by rinsing with distilled water. The smears were then prepared as mentioned above, further fixed by covering with a Schaudin's fluid containing 2 parts of saturated aqueous solution of $HgCl_2$ and 1 part of absolute ethanol for 5 minutes. Washing was done with water and placed in 1 N HCl at 60°C for 15 minutes. Usually one more washing with distilled water was needed before a staining with Giemsa stain at 37°C. The Giemsa stain solution was prepared by adding 2-3 drops of Giemsa stain (Sigma) in phosphate buffer

(0,272 % w/v Na_2HPO_4 and 0.237 % w/v KH_2PO_4 at pH 7). The stained smears, after further washing, showed reddish purple coloured nuclear structures.

12. Surface Morphological Study of Bacterial Cells by Scanning Electron Microscope

Bacterial cells were harvested and rinsed twice with distilled water by centrifugation (10,000 g, 10 min). Chemical fixation, as described by Kessil & Shih,(1976) and Pease (1964), was followed by suspending the bacterial cells firstly in 3 % glutaraldehyde and secondly in 2 % osmium tetroxide at 0 - 4°C for 30 minutes respectively. These two fixing solution were prepared by dissolving the chemicals in 0.1 M sodium cacodylate (pH 7) buffer. After chemical fixation, the cells were washed with sodium cacodylate buffer by centrifugation. Dehydration was done by passing the cells through graded concentrations of 25 %, 50 %, 70 %, 80 % and 100 % (v/v) ethanol. Bacterial cells were smeared on microscopic cover slid and mounted on copper specimen stubs for surface coating with gold. The coated specimens were examined with a scanning microscope (Jeol, JSM-35).

13. Analysis of Morphological (Filamentous) Alteration in E. coli

E. coli was cultured overnight in 1 % tryptone broth (TB) supplemented with 18-crown-6 ranging from 2.5×10^{-3} M to 3×10^{-2} M. The gross morphology of these cells was studied microscopically with the phase-contrast optics. The cells were recorded with a Kodak high contrast film (HC 135). These negatives were printed according to a standardized procedure and magnification to give an appropriate cell image in a 30 cm x 36 cm photographic paper. The length of the individual bacterium was measured with a curvimeter. This length was calibrated against the unit shown on a haemacytometer, which had been recorded through the same photographic procedures. The length of the smallest square on the haemacytometer is 0.05 mm.

14. Mechanical Separation of Filamentous Cells from Normal Cells

In all effective 18-crown-6 concentrations, an uneven cell length bacterial population was found. In order to separate the filamentous cells from the short nomal cells. The population was allowed to filter through a glass fiber membrane (Whatman, GF/C, pore

size 1.2 μm). Washing was repeated to remove residual normal cells with distilled water. The retained filamentous cells on the glass fiber membrane was collected by rinsing the membrane in distilled water. In practice, the filamentous cells were contaminated with fragments of glass fibres which could be removed by sedimentation at low speed centrifugation, 1500 g - 2000 g. The efficiency of the separation depended on many factors. Optimal yield was obtained with a washing at gentle flow rate containing five fold the volume of the original cell suspension at turbidity of 0.6.

15. Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Whole Cell Bacterial Protein

E. coli was cultured overnight (approximately 16 hours) in plain tryptone broth (as control) and in 1.5×10^{-2} M 18-crown-6 supplemented tryptone broth. The cells were harvested by centrifugation (10,000 g, 10 min) and washed twice with distilled water. Microscopically, filamentous E. coli cells were formed in tryptone broth with 1.5×10^{-2} M 18-crown-6. The cells were lysed either by sonication for 5 minutes with a sonicator cell disruptor (Heat System - Ultrasonics Inc., Model W200R), or by a 1 mg/ml lysozyme treatment (incubated at 37°C for 15 minutes).

The whole cell lysate samples with protein content about 5 mg/ml as determined by the modified Lowry method (Markwell et al., 1978), were diluted (1:1, v/v) with dissociating solution containing 0.125 M Tris-HCl buffer, pH 6.8, 2 % SDS, 2 % mercaptoethanol, 20 % glycerol and 0.004 % bromophenol blue. After boiling for 2 minutes, 20 µl of the samples were applied to each gel slot of 10 % or 12.5 % SDS polyacrylamide slab gel. Electrophoresis was performed at current of 3 mA per gel slot for about 2 hours, according to the method of Epstein et al. (1974). The gel apparatus used was constructed as described by Reid & Bieleski (1968). After the run, the gel was stained with 1 % coomassie brilliant blue (Sigma) in methanol for 2 hours. Destaining was followed by firstly immersing the gel in solvent containing 50 % (v/v) metanol and 7.5 % (v/v) acetic acid overnight and then transfered to solvent containing 5 % (v/v) methanol and 7.5 % (v/v) acetic acid.

Molecular weight of the protein subunits was determined in relation to their corresponding motilities upon the SDS - PAGE with both the sample and the commercial available protein markers of molecular weight ranging from 10,000 to 70,000 (Sigma, MW-SDS-70).

III. RESULTS

1. Complexability of Crown Ether towards Alkali Metal Ions

Picrate anion is mainly soluble in aqueous solution and its presence in the organic solvent depends on its formation with the alkali metal ion as an ion pair which in turn is complexed with the crown ether. On account of the quantitative nature of the ion pair formation, the amount of picrate present in the organic phase has been used as a measure of the complexability of the crown ether with the alkali metal ion (Pedersen, 1968; Eisenman et al.,1968; Frensdorff, 1971; Ng, 1979; Wong et al., 1974). Picrate salt is chosen because of its large structure and highly polarizable property that render an extraordinary stability in the picrate-crown complex (Wong et al., 1974a). Besides, an extra merit of the yellowish picrate complex is its high absorptivity in the visible range.

The absorption maxima and the molar absorptivities of various crown ether-potassium picrate complexes in chloroform solution were determined and listed in Table 3. In the same table, some data from Ng (1979) were incorporated for comparison. The absorption spectra of various crown ether-picrate salt complexes in the chloroform

Table 3. Absorption Maxima and Molar Absorptivities of Crown Ether-Picrate Salt Complexes in Chloroform at 20°C

Crown Ether	Complexing Salt	Absorption Maxima λ_{max}(nm)	Molar Absorptivity ε (x10^4 $M^{-1}cm^{-1}$)
18-Crown-6	KPi	365	1.58
Benzo-18-crown-6	KPi	365	1.50
4'-Methyl-benzo-18-crown-6	KPi	365(365)*	1.47(1.7)*
	NaPi*	362	1.6
	RbPi*	365	1.7
	CsPi*	367	1.8
Dicyclohexyl-18-crown-6	KPi	365	1.58
4'-Methyl-benzo-15-crown-5*	KPi	365	1.7
	NaPi	356	1.6
	RbPi	365	1.7
	CsPi	364	1.8

*Data taken from Ng (1979)

layer after solvent extraction were collected in Appendix I.and the corresponding absorbance at absorption maximum were listed in Table 4. It was found that the absorption maxima of the crown ether complexes studied were confined to the wavelength in the range of 352 nm - 367 nm. It has been suggested that the absorption maximum depends on the mode of the complexation, and that at least two types of ion pairs can be formed in low polarity medium, such as chloroform, i.e. the crown-complexed tight ion pair and the crown separated ion pair (Wong et al., 1974). The tight ion pair has its cation located at the centre of the crown cavity forming 1:1 complex which gives absorption maxima around 350 - 370 nm. With the loose type, the cation is located in between two crown molecules forming a sandwiched complex, with a crown ether and cation ratio of 2:1. This latter complex has its absorption maximum at a longer wavelength,around 370-380 nm. According to this criterion, all the complexes involved in this study as shown in Table 4, were tight 1:1 ion pair complex type. Furthermore, similar molar absorptivity values were found within the same type of ion-pair complex, as illustrated in Table 3.

In the solvent extraction method, the overall reaction of crown ether with picrate salt can be described

Table 4. The Absorbance of Crown Ether-Picrate Salt Complexes Extracted in the Organic Phase*

Crown Ether	Absorbance of Crown Ether-Picrate Salt Complex[a]				
	K^+	Na^+	Rb^+	Cs^+	Li^+
15-Crown-5	0.228(355)‡	0.805(352)	0.163(354)	0.130(358)	0.082(352)
18-Crown-6	3.545(361)	0.285(356)	3.424(360)	1.960(365)	0.130(356)
Benzo-18-crown-6	2.765(361)	0.275(354)	1.976(360)	0.808(365)	0.072(352)
4'-Methyl-benzo-18-crown-6	3.185(363)	0.287(360)	2.272(362)	1.020(365)	0.072(352)
Dicyclohexyl-18-crown-6	3.645(363)	0.670(360)	3.152(363)	1.928(367)	0.104(358)
Control (without crown)[b]	0.015(356)	0.070(340)	0.082(344)	0.048(356)	0.056(352)

*The amount of complex in the organic phase was determined by the solvent extraction method as described in Materials and Methods. In the binary aqueous-chloroform system, the initial concentration of various chemical components were:
Crown ether ; 2.5×10^{-4}M,
Picric acid ; 3.93×10^{-4}M,
and the alkali metal chloride ; 0.1M

‡Absorption maximum (λ max) of the corresponding complex, in unit of nm.

[a]The corresponding spectra were provided in Appendix I.

[b]Note a small background in the absence of crown ether with a distinct absorption maximum.

as

$$M^{+}_{aq} + Cr_{org} + Pi^{-}_{aq} \xrightleftharpoons{K_e} M^{+},Cr,Pi^{-}_{org}$$

according to Eisenman et al. (1968). In the equation, M^{+}_{aq} and Pi^{-}_{aq} denote the alkali cation and picrate anion in aqueous phase, M^{+},Cr,Pi^{-}_{org} and Cr_{org} denote the crown-picrate salt complex and non-complexed crown ether in the organic phase. The overall extraction process involved the transfer of alkali cation and picrate anion from the aqueous phase into the organic phase by the formation of the ion pair $M^{+}Pi^{-}$,

$$M^{+}_{aq} + Pi^{-}_{aq} \xrightleftharpoons{K} M^{+}Pi^{-}_{org}$$

and the complexation between the ion pair and the crown ether in the organic phase (Wong et al., 1977),

$$M^{+}Pi^{-}_{org} + Cr_{org} \xrightleftharpoons{K_c} M^{+},Cr,Pi^{-}_{org}$$

The overall extraction equilibrium constant (K_e) is then given as

$$K_e = KK_c$$

If the alkali metal ion forms a 1:1 complex with the crown ether and the concentration of the picrate salt in the aqueous phase is much greater than the initial

crown ether concentration in the organic layer, the extraction equilibrium constant, K_e, can be calculated by the following expression,

$$K_e = \frac{\left[MCrPi_{org}\right]}{\gamma\pm^2 \left[M^+_{aq}\right]\left[Pi^-_{aq}\right]\left\{\left[Cr_{org}\right]_o - \left[MCrPi_{org}\right]\right\}}$$

where $\gamma\pm$ is the mean activity coefficient of the picrate salt in water and $\left[Cr_{org}\right]_o$, $\left[MCrPi_{org}\right]$ are the initial crown ether concentration and crown ether-picrate salt complex concentration in organic phase, respectively. At low component concentrations, γ can be considered to be approaching unity. Experimentally, the concentration of the crown ether-picrate salt complex in organic phase is derived from its corresponding absorbance. With the above concentrations, the extraction equilibrium constants (K_e) were calculated in Table 5. This K_e value represents the complexability of the crown ether towards the alkali metal ion.

In addition, the magnitudes of the K_e values of the crown ether with each of the corresponding metal ions reflect the selectivity of the crown ether towards the alkali metal species. Data taken from Table 5 were recalculated to show this property and given in Table 6. It was apparent that except 15-crown-5, all 18-crown-6 members exhibited higher complexability to potassium ion.

Table 5. Extraction Equilibrium Constants (K_e) of Crown Ether with Alkali Metal Picrates Salts in an Aqueous-Chloroform System at 20°C

Crown Ether	Extraction Equilibrium Constant* K_e ($\times 10^4 M^{-2}$)				
	KPi	NaPi	RbPi	CsPi	LiPi
15-Crown-5	0.15	0.75	0.11	0.09	0.05
18-Crown-6	51.99	0.21	36.91	3.66	0.09
Benzo-18-crown-6	13.44	0.21	4.26	0.81	0.05
4'-Methyl-benzo-18-crown-6	36.91(23.00)[a]	0.23(0.23)[a]	6.78(6.90)‡	1.19(1.40)‡	0.05
Dicyclohexyl-18-crown-6	73.65	0.58	20.42	3.52	0.07

*The molar absorptivities (ε) employed were as indicated in Table 3. When metal ions other than potassium were used, the value were still assumed to be equal to their potassium counterparts. Similarly, value of 15-crown-5 complex was based on that determined for the 18-crown-6 complex.

[a]Data taken from Wong et al., 1977.

‡Data taken from Ng, 1979.

Table 6. Selectivity* of Crown Ethers towards Alkali Metal Ions

Crown Ether	Selectivity	Ratio
15-Crown-5	Na > K > Rb > Cs > Li	15.0 : 3.2 : 2.2 : 1.8 : 1.0
18-Crown-6	K > Rb > Cs > Na > Li	577.7 : 410.1 : 40.7 : 2.3 : 1.0
Benzo-18-crown-6	K > Rb > Cs > Na > Li	268.8 : 85.2 : 16.2 : 4.2 : 1.0
4'-Methyl-benzo-18-crown-6	K > Rb > Cs > Na > Li	738.2 : 135.6 : 23.8 : 4.6 : 1.0
Dicyclohexyl-18-crown-6	K > Rb > Cs > Na > Li	1052.1 : 291.7 : 50.3 : 8.3 : 1.0

*Selectivity was calculated from data given in Table 5 and a trend was arranged correspondingly.

Since potassium and sodium are common physiological alkali metal ions and their transport through biological membrane have received much attention recently, the selectivity of this couple and the frequently used potassium substitute, rubidium, were separately compared in Table 7. It was clearly shown that the parent 18-crown-6 had the highest selectivity for potassium and the aromatic substituted 18-crown-6 was the least selective one among the 18-crown-6 family. The complexability of crown ether to rubidium was somewhat smaller than that of potassium, as indicated by the ratio in the last column.

Crown ether, being lipid soluble, seems to be able of incorporating into the membrane fraction of the biological system and thus interfere with the transport of alkali metal ions. Wong et al. have studied the ion transport efficiency of crown ether in an artificial chloroform membrane system and suggest that the crown ether facilitated ion transport rate is proportional to the extraction equilibrium constant of the corresponding crown-cation complex (Wong et al., 1974). This is in essence similar to that suggested by Ward (1970). According to this consideration, the efficiency of crown ether facilitated ion transport can be estimated by its corresponding K_e value. An attempt to illustrate the

Table 7. A Comparison of the Crown Ether Selectivity in Different Alkali Metal Pairs*

Crown Ether	Template Selectivity		
	K^+/Na^+	Rb^+/Na^+	K^+/Rb^{+a}
15-Crown-5	0.2	0.1	1.4
18-Crown-6	247.6	175.7	1.4
Benzo-18-crown-6	64.0	20.3	3.1
4'-Methyl-benzo-18-crown-6	160.5	29.5	5.4
Dicyclohexyl-18-crown-6	127.0	35.2	3.6

*These ratios were calculated from the extraction equilibrium constants in Table 5.

[a]Rubidium has been used in many physiological studies as a substitute for potassium. This ion substitution is indispensible in radioactive labelled experiments where ^{86}Rb is frequently used. In this column, a comparison of potassium versus rubidium was given to show the similarity of the two ions in complex with various crown ethers.

transport efficiency among the various crown ether for three commonly studied alkali metal ion was based on comparison of K_e values (Table 8). It was shown that the 18-crown-6 family facilitated potassium transport more efficiently than 15-crown-5. It was worthwhile to note that although 15-crown-5 had a high selectivity for sodium, its sodium ion transport efficiency, however, was not that much differed from the 18-crown-6 family. And that as transport efficiency was concerned, dicyclohexyl-18-crown-6 appeared to be the most efficient one for potassium ion transport.

2. Toxicity of Crown Ether on E. coli

The MIC susceptibility test, which is usually as a preliminary test of chemical toxicity before investigating their mode of action on bacterial cells, showed that the 18-crown-6 family studied exhibited similar toxicity in E. coli, While 15-crown-5 was less toxic (ten folds less than 18-crown-6) as indicated by the trend of turbidity decrease (Fig. 2) as well as the MIC and LD_{50} values determined (Table 9).

3. Toxicity of 18-crown-6 to Various Microorganisms

All bacterial species t[illegible]ted except Halobacterium

Table 8. A Comparison of the Ion Transport Efficiency in the Presence of Crown Ethers

Crown Ether	Ion Transport Efficiciency*		
	K	Rb	Na
15-Crown-5	1.0	1.0	3.6
18-Crown-6	324.9	335.5	1.0
Benzo-18-crown-6	84.0	38.7	1.0
4'-Methyl-benzo-18-crown-6	230.7	61.1	1.1
Dicyclohexyl-18-crown-6	460.3	185.6	2.8

*The comparison given here is made convenient only for comparison among the crown ether complex of the same alkali metal ion; in case where interionic comparison is needed, refer to Table 5.

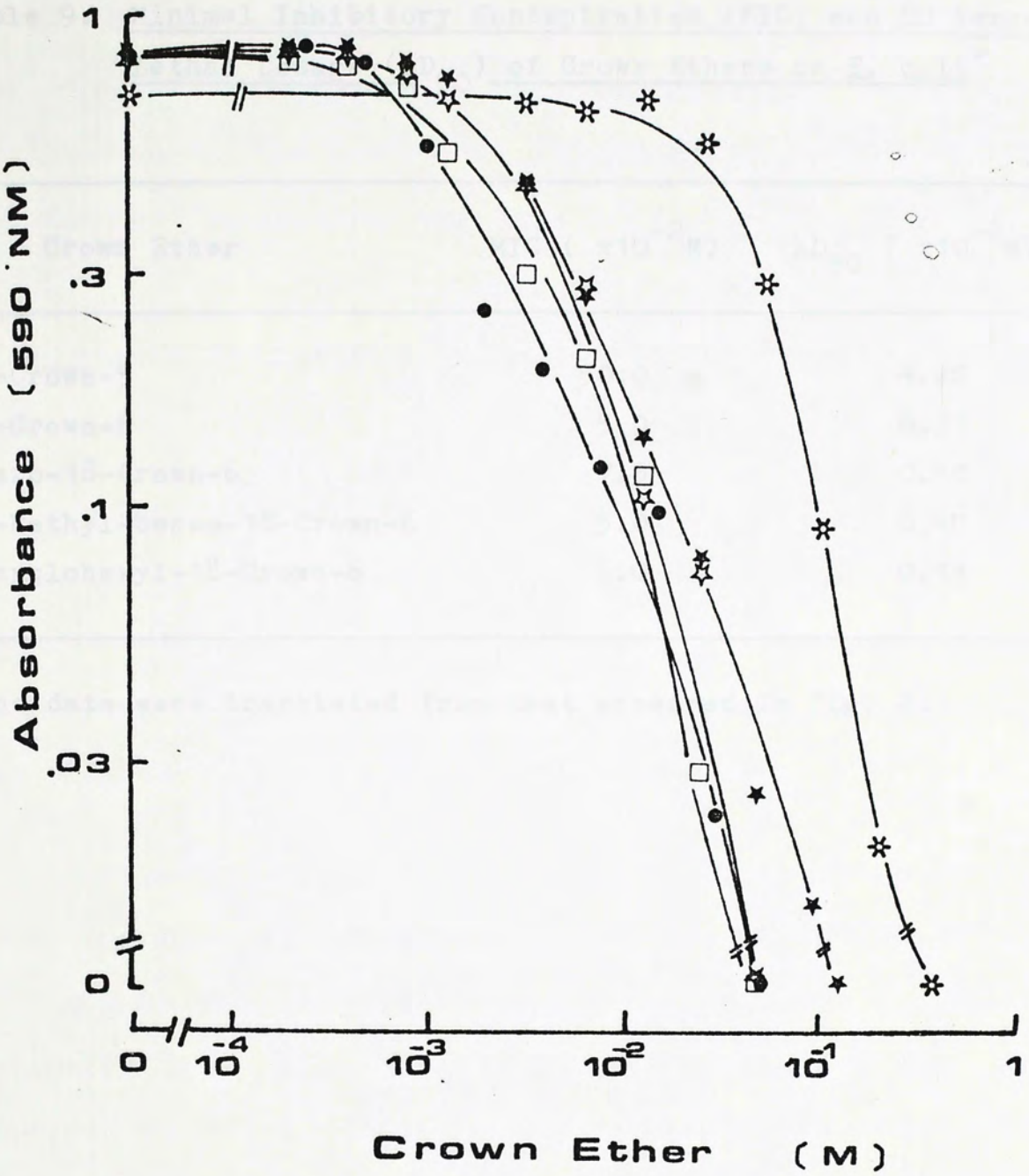


Figure 2. MIC Test of Crown Ether Toxicity in E. coli

Turbidity measurement was on overnight culture (approximately 16 hours). Symbols for crown ethers : (✲) 15-crown-5, (□) 18-crown-6, (✡) benzo-18-crown-6, (✶) 4'-methyl-benzo-18-crown-6 and (●) dicyclohexyl-18-crown-6.

Table 9. Minimal Inhibitory Concentration (MIC) and 50 Percent Lethal Dosage (LD_{50}) of Crown Ethers on E. coli*

Crown Ether	MIC ($x10^{-2}M$)	LD_{50} ($x10^{-2}M$)
15-Crown-5	50.0	4.20
18-Crown-6	5.0	0.21
Benzo-18-Crown-6	7.5	0.40
4'-Methyl-benzo-18-Crown-6	5.0	0.40
Dicyclohexyl-18-Crown-6	5.0	0.13

*The data were translated from that appeared in Fig. 2.

showed more or less similar susceptibility to 18-crown-6 toxicity, while halophilic Halobacterium was more sensitive to 18-crown-6. The protozoa, Tetrahymena, nevertheless, was the most sensitive one (Fig. 3 and Table 10).

4. Effect of Polyethers on the Morphology and Motility of E. coli

Neither the crown ether nor the polyethylene glycol, tested at their highest concentration to about 10^{-2} M showed any significant morphological alterations in non-growing E. coli. As recorded in Table 11, the cells were observed to be intact, and fully motile.

Polyethylene glycol, at its highest concentration used, had created none-so-ever adverse effect on the motility as well as the morphology of growing E. coli. Seemingly, only a slight reduction in motility was observed in cultures supplemented with crown ether at comparatively high concentration (5×10^{-3} M - 10^{-2} M). This motility reduction was more severe in 18-crown-6 supplemented media, apparently because a morphological effect was also noted (see later studies)

Figure 3. MIC Test of 18-Crown-6 Toxicity on Various Microorganisms

Symbols for microorganism species :

(✶) Salmonella typhimurium,

(✡) Streptococcus,

(□) Staphylococcus coagulate (+),

(✥) Staphylococcus coagulate (-),

(✱) Corynebacterium,

(●) Bacillus,

(✽) Halobacterium cutiritium,

(*) Tetrahymena thermophila.

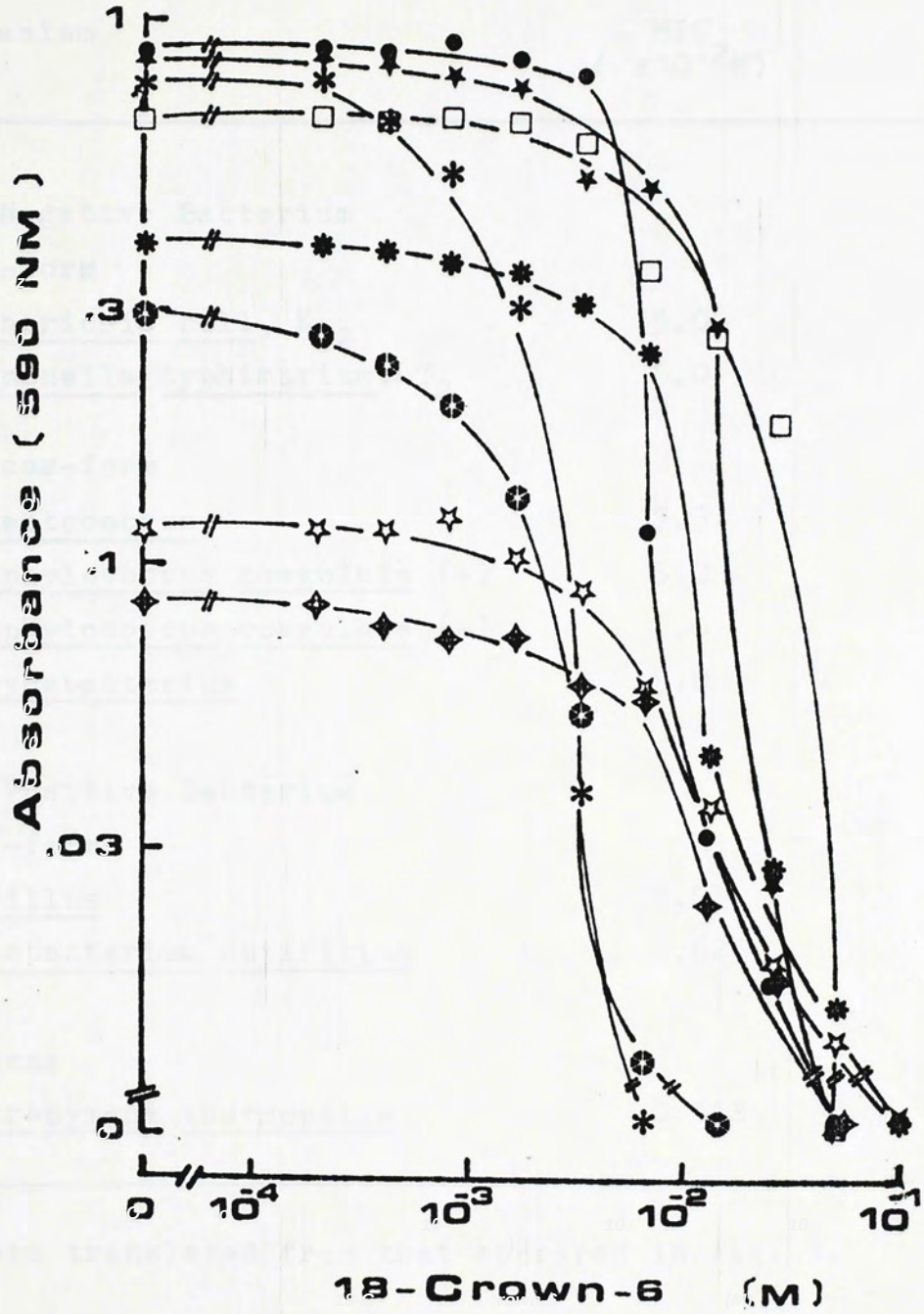

Absorbance (590 NM)
1
.3
.1
.03
0
0
10^{-4}
10^{-3}
10^{-2}
10^{-1}
18-Crown-6 (M)

Table 10. MIC and LD_{50} of 18-Crown-6 on Various Microorganisms*

Microorganism	MIC ($\times 10^{-2}$M)	LD_{50} ($\times 10^{-2}$M)
A. Gram-Negative Bacterium		
a. Rod-form		
Escherichia coli, K_{12}	5.0	0.21
Salmonella typhimurium, LT_2	5.0	0.63
b. Coccus-form		
Streptococcus	7.5	0.71
Staphylococcus coagulate (+)	5.0	0.91
Staphylococcus coagulate (-)	5.0	0.79
Corynebacterium	5.0	0.71
B. Gram-Positive Bacterium		
a. Rod-form		
Bacillus	5.0	0.45
Halobacterium cutiritium	0.625	0.14
C. Protozoa		
Tetrahymena thermophila	0.312	0.13

*Data were translated from that appeared in Fig. 3.

Table 11. Effect of Polyether on the Morphology and Motility of Escherichia coli

Crown Ether		Escherichia coli			
		Non-Growth State		Growing State	
Type	Concentration ($x10^{-2}M$)	Morphology	Motility	Morphology	Motility
15-Crown-5	1	intact, rod shape	motile	intact, rod shape	motile
18-Crown-6	1	"	"	intact, filamentous	slow movement
Benzo-18-crown-6	1	"	"	intact, rod shape	slightly slower movement
4'-Methyl-benzo-18-crown-6	0.5	"	"	"	"
Dicyclohexyl-18-crown-6	0.5	"	"	"	"
Polyethylene 200	1	"	"	"	motile
Polyethylene 400	1	"	"	"	motile

5. Effect of Polyethers on the Bacterial Growth Curve

The presence of crown ether in growth medium affected the three major phases in the bacterial growth curve with an appearance of a lag period, an occasional decrease in the slope at the logarithmic phase and an early development of the stationary phase at a lower microbial population. All these effects were concentration dependant, as shown in Fig. 4 for sodium tryptone broth (NaTB) bacterial cultures. The potassium phosphate minimal medium (KMM) cultures gave essentially similar pattern except that no attempt was made to obtain the growth yield at the initial stationary phase due to excessively long incubation hours (Fig. 5). In NaTB cultures, equal molar of the 18-crown-6 members compared at 5×10^{-3} M showed that dicyclohexyl-18-crown-6 produced the longest lag (Table 12). Except at high concentration of 18-crown-6 cultures, the doubling time was practically unchanged. The growth yield was in general affected by the presence of crown ether and as compared at 5×10^{-3} M of each crown ether, dicyclohexyl-18-crown-6 also exhibited the largest reduction. A comparison of the two effects which had been shifted significantly, namely, the lag period and the growth yield was given in Fig. 6. Obviously, the two effects were parallel to each other.

Figure 4. Effect of Crown Ether on E. coli Growth Curve in Sodium Tryptone Broth.

Panel A : 18-crown-6, (✡) tryptone alone, (●) 10^{-3} M crown ether, (✱) 5 x 10^{-3} M crown ether, (✦) 10^{-2} M crown ether. Panel B : benzo-18-crown-6, same symbols as in A were used to represent the chemical concentrations. Panel C : 4'-methyl-benzo-18-crown-6, (✡) tryptone alone, (●) 10^{-4} M crown ether, (✱) 10^{-3} M crown ether, (✦) 5 x 10^{-3} M crown ether and panel D : dicyclohexyl-18-crown-6, (✡) tryptone alone, (●) 5.24 x 10^{-4} M crown ether, (✱) 8.26 x 10^{-4} M crown ether, (✦) 5.24 x 10^{-3} M crown ether.

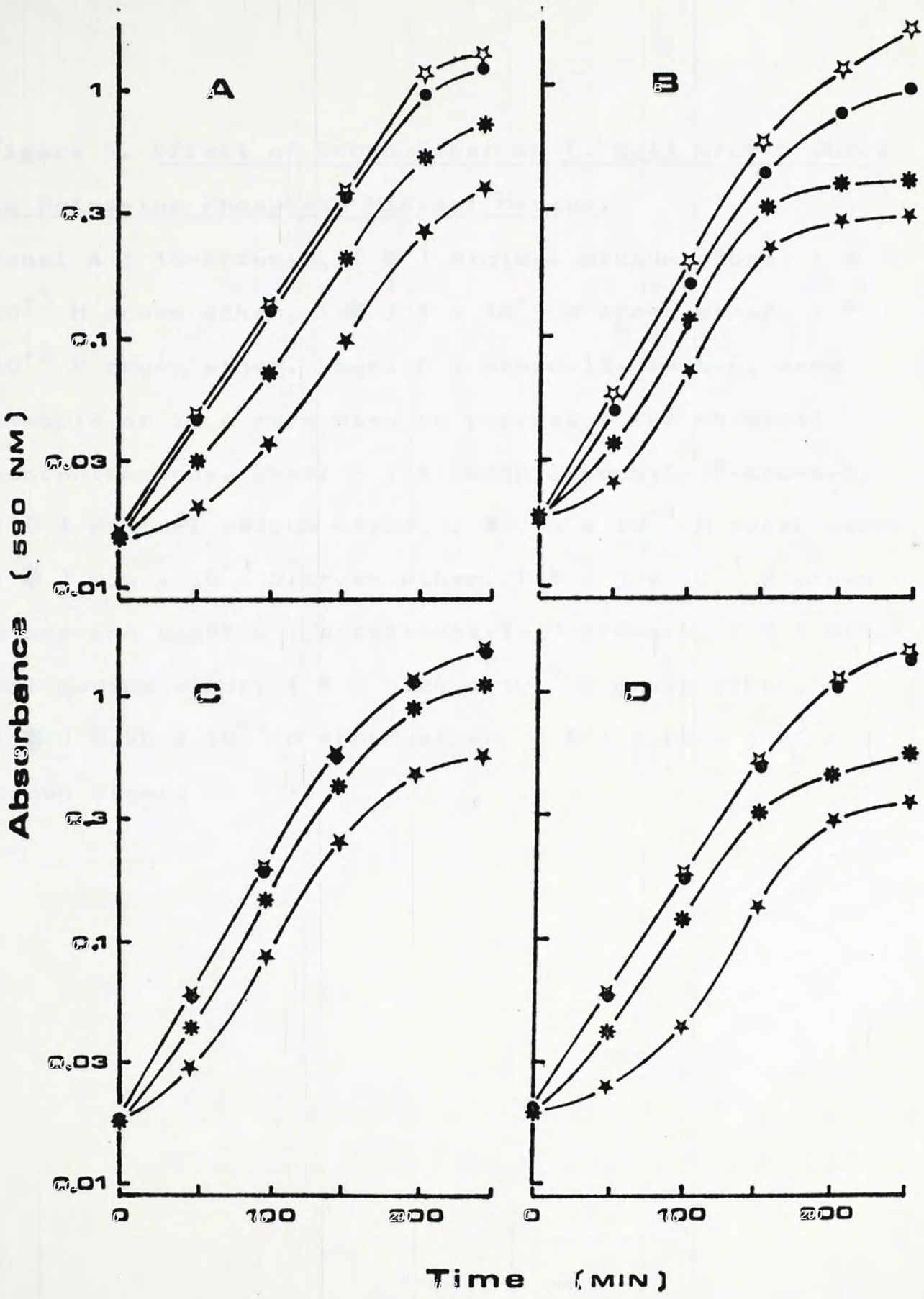

A
B
C
D
1
0.3
0.1
0.03
0.01
Absorbance (590 NM)
0
100
200
Time (MIN)

Figure 5. Effect of Crown Ether on E. coli Growth Curve in Potassium Phosphate Minimal Medium.

Panel A : 18-crown-6, (✡) minimal medium alone, (●) 10^{-3} M crown ether, (✱) 5 x 10^{-3} M crown ether, (✦) 10^{-2} M crown ether. Panel B : benzo-18-crown-6, same symbols as in A were used to represent the chemical concentrations. Panel C : 4'-methyl-benzyl-18-crown-6, (✡) minimal medium alone, (●) 5 x 10^{-4} M crown ether, (✱) 2.5 x 10^{-3} M crown ether, (✦) 5 x 10^{-3} M crown e ether and panel D : dicyclohexyl-18-crown-6, (✡) minimal medium alone, (●) 5.24 x 10^{-4} M crown ether, (✱) 8.26 x 10^{-4} M crown ether, (✦) 5.24 x 10^{-3} M crown ether.

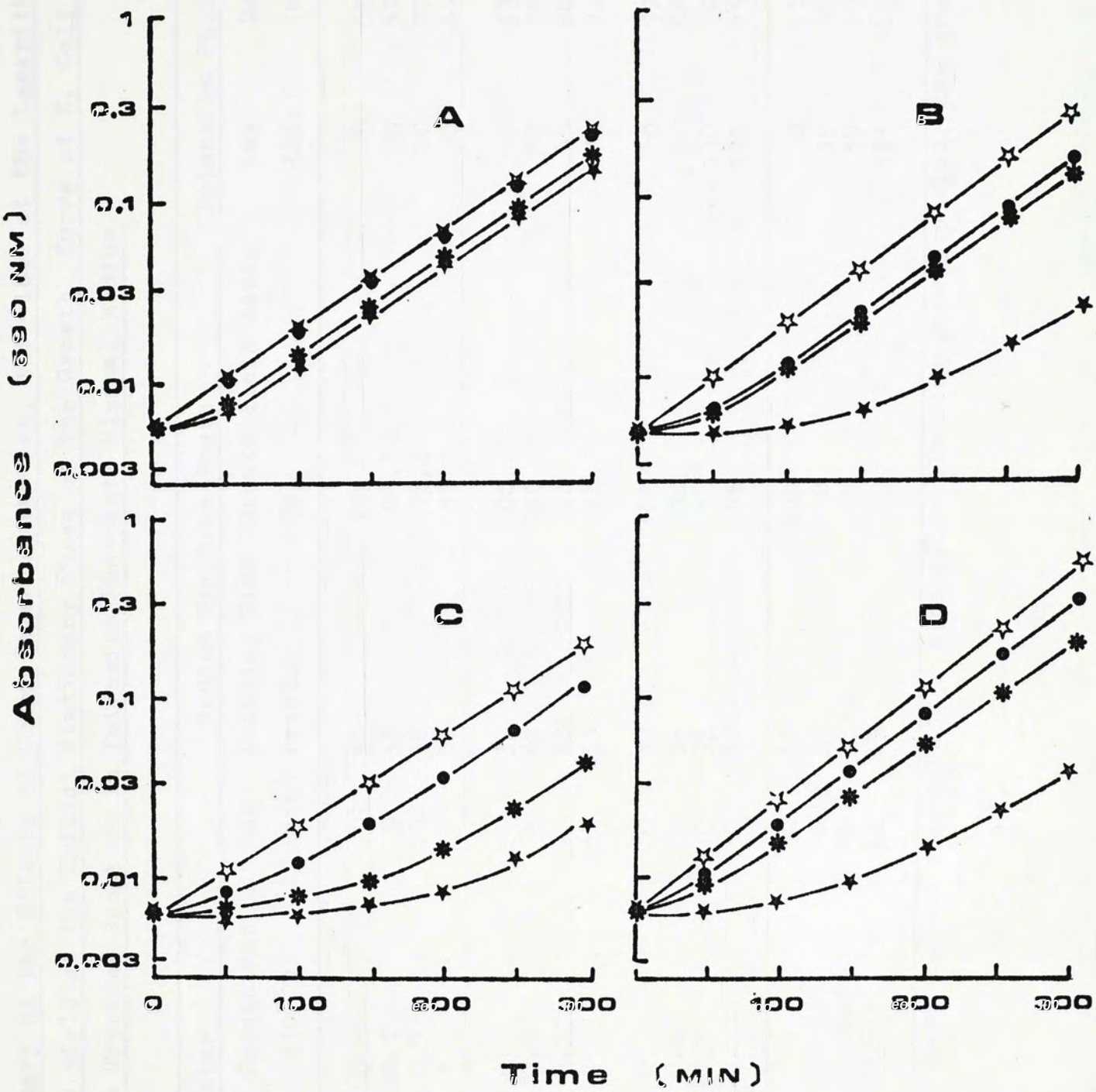

A
B
C
D
1
0.3
0.1
0.03
0.01
0.003
0
100
200
300
Absorbance (600 NM)
Time (MIN)

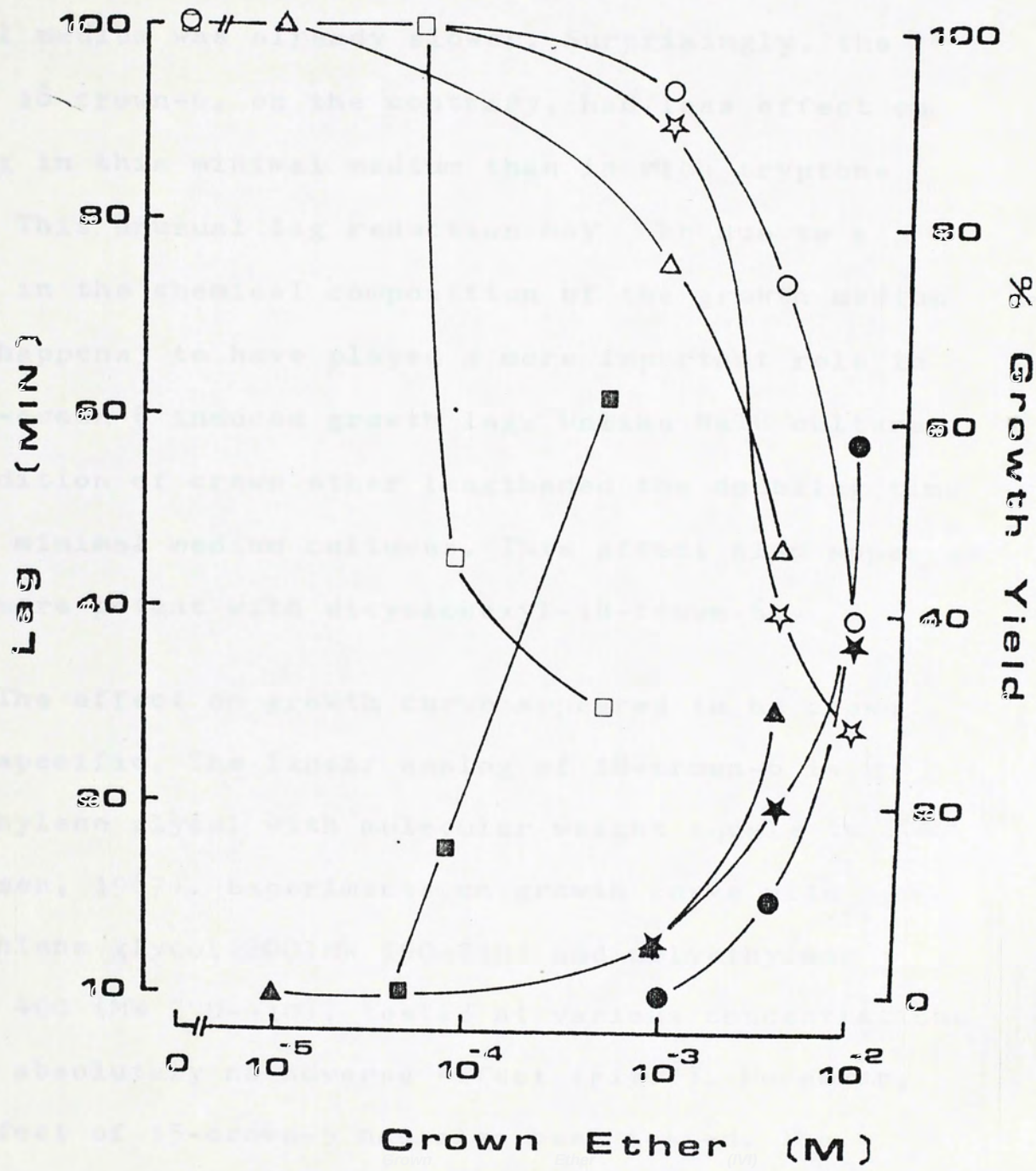
Table 12. A Summary of the Effects of Crown Ether on the Lag, the Slope at the Logarithmic Phase, and the Growth yield at the Initial Stationary Phase of the Growth Curve of E. Coli Grown either in Sodium Tryptone Broth or in Potassium Phosphate Minimal Medium

Crown Ether		Sodium Tryptone Broth			Potassium Phosphate Minimal Medium*	
Type	Concentration ($x10^{-2}M$)	Lag (min)	Doubling Time (min)	Growth Yield Ratio (%)	Lag (min)	Doubling Time (min)
18-crown-6	0	0	33	100	0	55
	0.1	0	33	93.3	0	57
	0.5	10	36	73.3	20	57
	1	58	37	39.3	30	61
benzo-18-crown-6	0	0	32	100	0	53
	0.1	5	32	70	40	55
	0.5	20	32	40	45	60
	1	37	33	28	135	72
4'-methyl-benzo-18-crown-6	0	0	32	100	0	57
	0.01	0	32	100	40	63
	0.1	10	32	76	110	70
	0.5	30	32	46	190	73
dicyclohexyl-18-crown-6	0	0	33	100	0	53
	0.0524	0	33	100	15	56
	0.0826	15	33	45	35	65
	0.524	62	33	30	133	69

*Since Stationary phase was not reached after a considerable long growth period, the growth yield was not calculated.

Figure 6. A Comparison of the Crown Ether Induced Lag and Growth Yield Reduction in Growth Curve of E. coli Tryptone Broth Culture.

Solid symbols represent the lag and open symbols represent growth yield percentage. (● , ○) 18-crown-6, (★ , ☆) benzo-18-crown-6, (▲ , △) 4'-methyl-benzo-18-crown-6 and (■ , □) dicyclohexyl-18-crown-6. Data were taken from Table 12.


100
80
60
40
20
10
Lag (MIN)
100
80
60
40
20
0
% Growth Yield
0
10^-5
10^-4
10^-3
10^-2
Crown Ether (M)

For KMM cultures, the effect on the lag was more extreme in the three substituted 18-crown-6. This might be expected since the growth rate of E. coli in this minimal medium was already slower. Surprisingly, the parent 18-crown-6, on the contrary, had less effect on the lag in this minimal medium than in rich tryptone broth. This unusual lag reduction may be due to a change in the chemical composition of the growth medium which happens to have played a more important role in the 18-crown-6 induced growth lag. Unlike NaTB cultures, the addition of crown ether lengthened the doubling time of the minimal medium cultures. This effect also appeared to be more potent with dicyclohexyl-18-crown-6.

The effect on growth curve appeared to be crown ether specific. The linear analog of 18-crown-6 is hexaethylene glycol with molecular weight equals to 282 (Pedersen, 1967). Experiments on growth curve with polyethlene glycol 200(MW 190-210) and polyethylene glycol 400 (MW 390-410), tested at various concentrations showed absolutely no adverse effect (Fig.7). Moreover, the effect of 15-crown-5 had also been tested. The experimental result showed that this compound, at a concentration which 18-crown-6 had already shown obvious effect, however, indicated no significant effect on

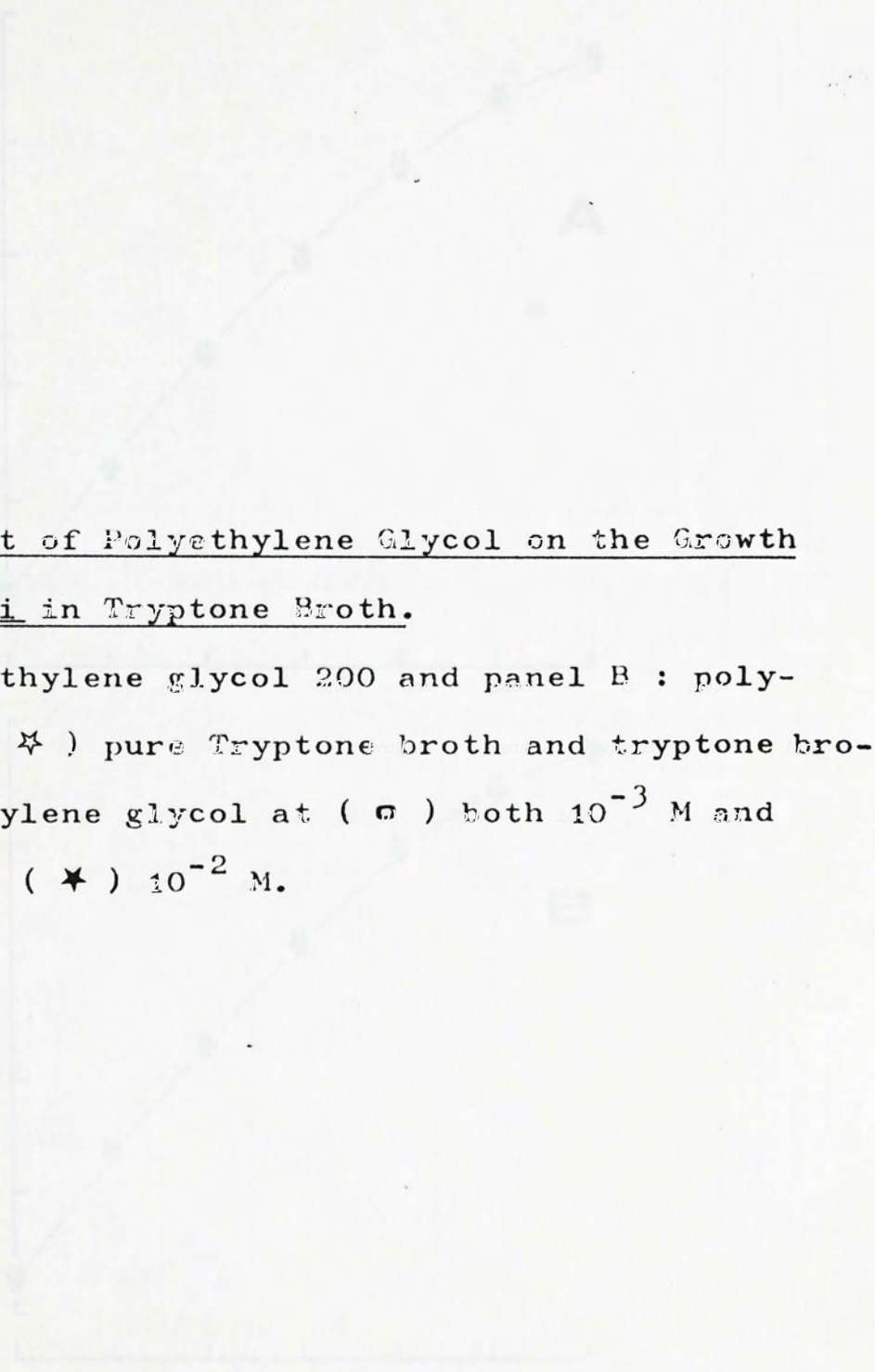
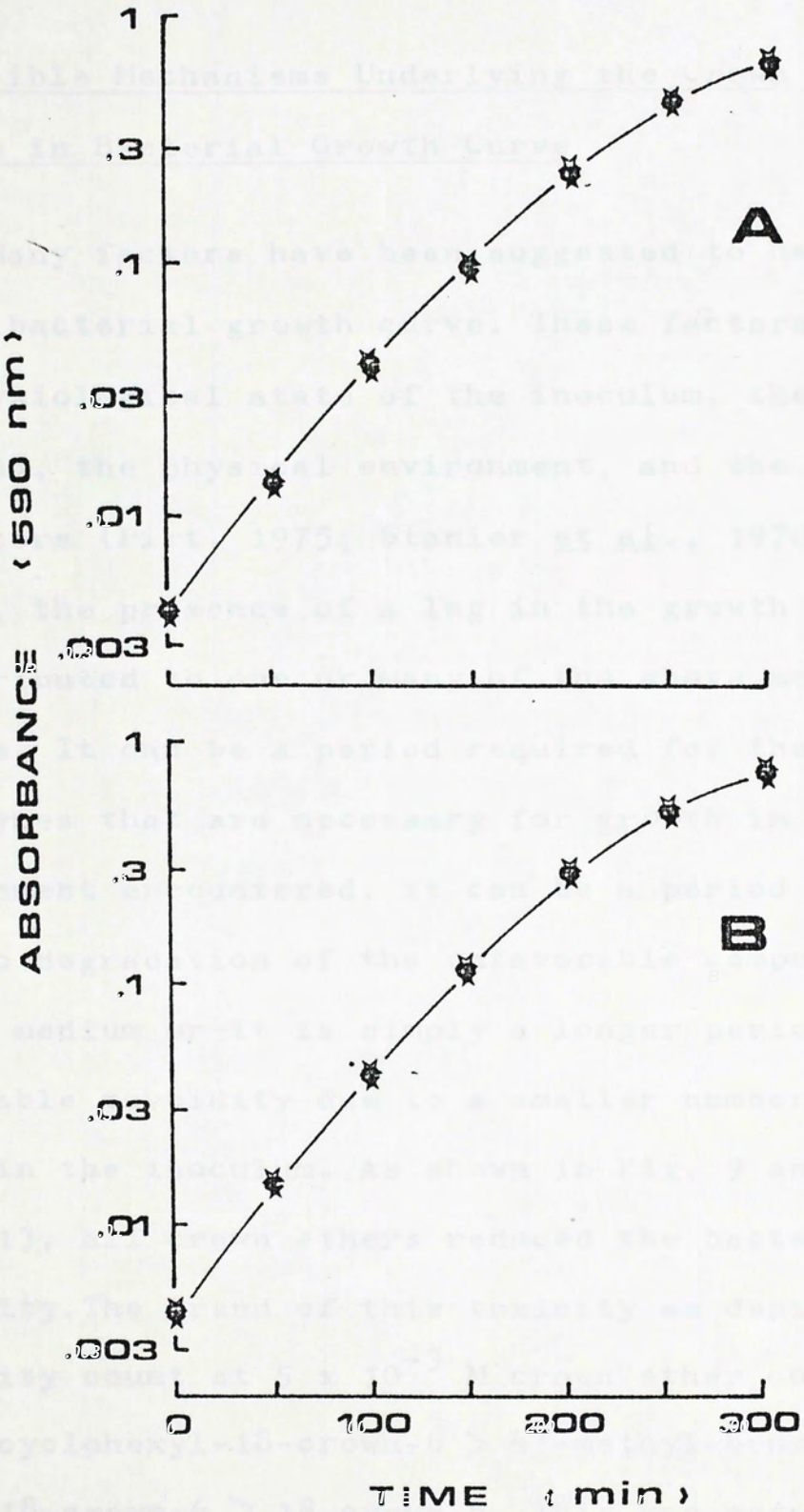


Figure 7. Effect of Polyethylene Glycol on the Growth Curve of E. coli in Tryptone Broth.

Panel A : polyethylene glycol 200 and panel B : polyethylene 400. (☆) pure Tryptone broth and tryptone broth with polyethylene glycol at (□) both 10^{-3} M and 5×10^{-3} M, and (★) 10^{-2} M.


1
,3
,1
,03
,01
,003
ABSORBANCE (590 nm)
A
B
0
100
200
300
TIME (min)

growth curve (Fig. 8).

6. Possible Mechanisms Underlying the Crown Ether Induced Changes in Bacterial Growth Curve

Many factors have been suggested to have an effect on the bacterial growth curve. These factors include the physiological state of the inoculum, the nutrition employed, the physical environment, and the presence of inhibitors (Pirt, 1975; Stanier *et al.*, 1970). As a result, the presence of a lag in the growth curve can be attributed to one or many of the above mentioned factors. It can be a period required for the induction of enzymes that are necessary for growth in the new environment encountered, it can be a period required for the degradation of the unfavorable compounds present in the medium or it is simply a longer period to reach measurable turbidity due to a smaller number of viable cells in the inoculum. As shown in Fig. 9 and listed in Table 13, all crown ethers reduced the bacterial cell viability. The trend of this toxicity as depicted in viability count at 5×10^{-3} M crown ether concentration was dicyclohexyl-18-crown-6 > 4'-methyl-benzo-18-crown-6, benzo-18-crown-6 > 18-crown-6. This was essentially the same trend as observed in their corresponding potency

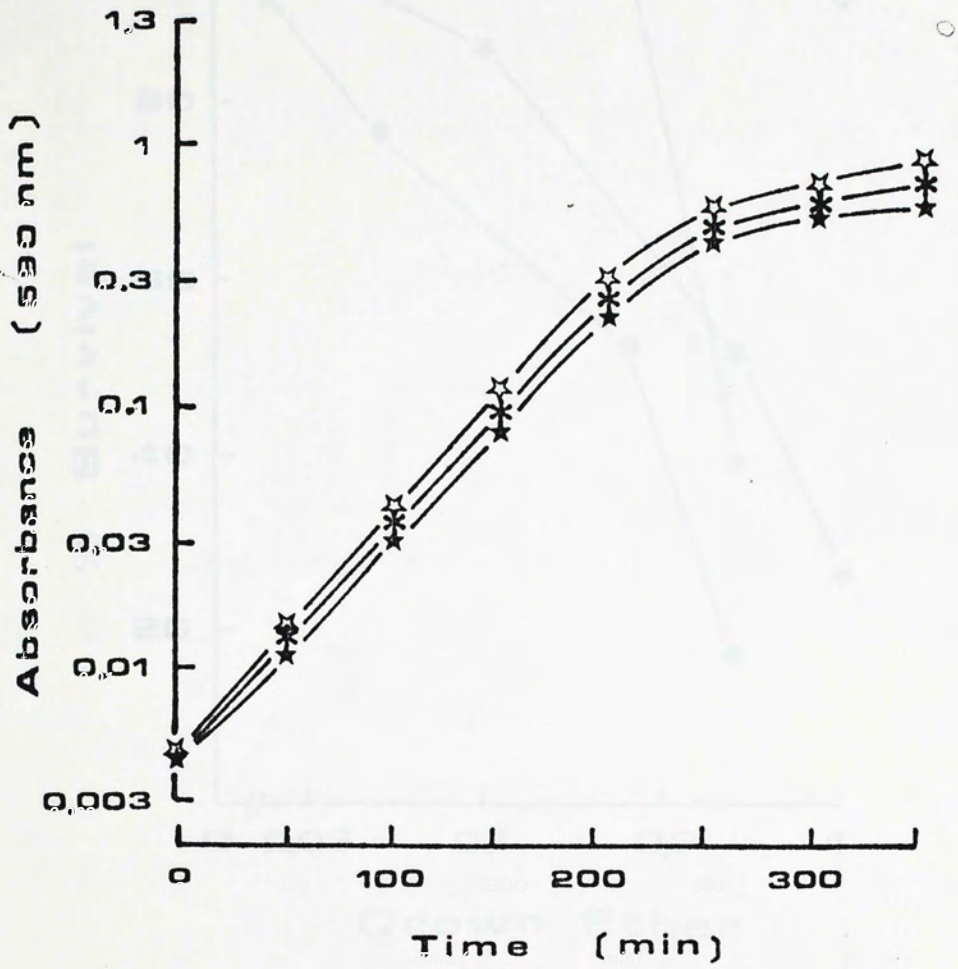


Figure 8. Effect of 15-Crown-5 on the Growth Curve of E. coli in Tryptone Broth.

(☆) Pure tryptone broth, and tryptone broth with 15-crown-5 at both (*) 10^{-3} M and 5 x 10^{-3} M, and (★) 10^{-2} M.

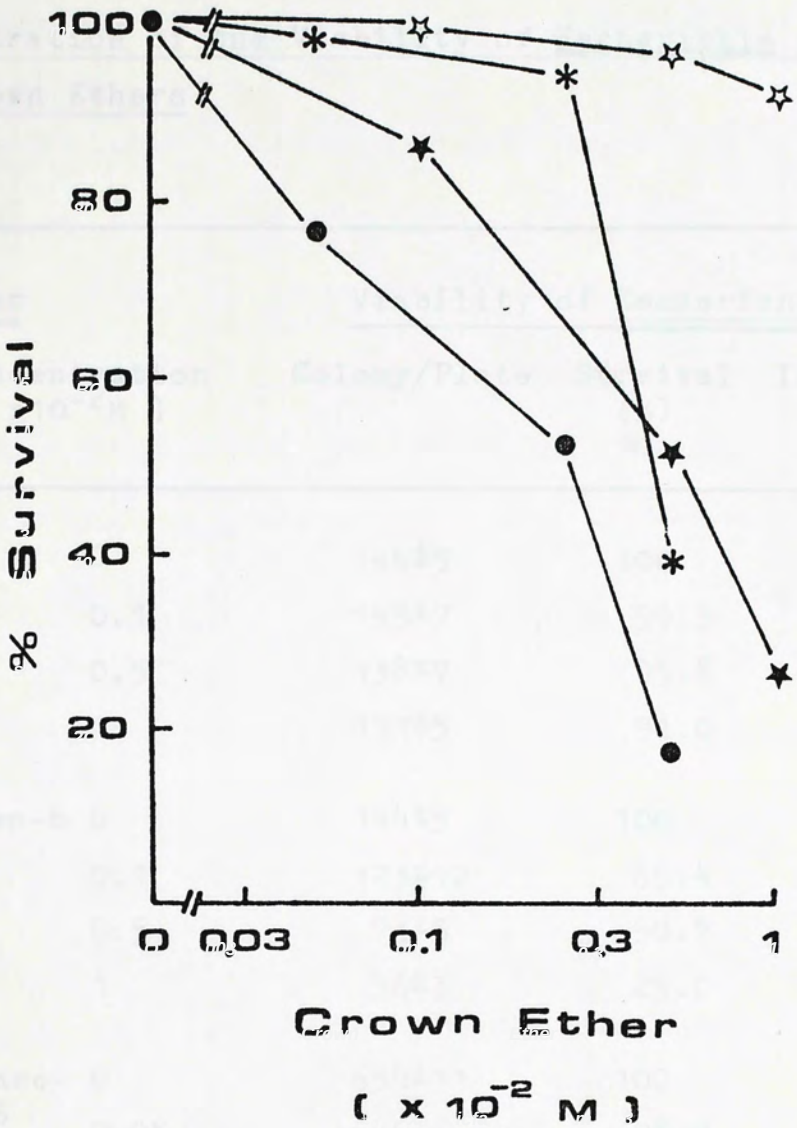


Figure 9. Titration of the Viability of E. coli in Crown Ethers.

Standard viability test method was employed as described in Materials and Methods. (☆) 18-crown-6, (★) benzo-18-crown-6, (*) 4'-methyl-benzo-18-crown-6 and (●) dicyclohexyl-18-crown-6. Data were taken from Table 13.

Table 13. Titration of the Viability of Escherichia coli in Crown Ethers

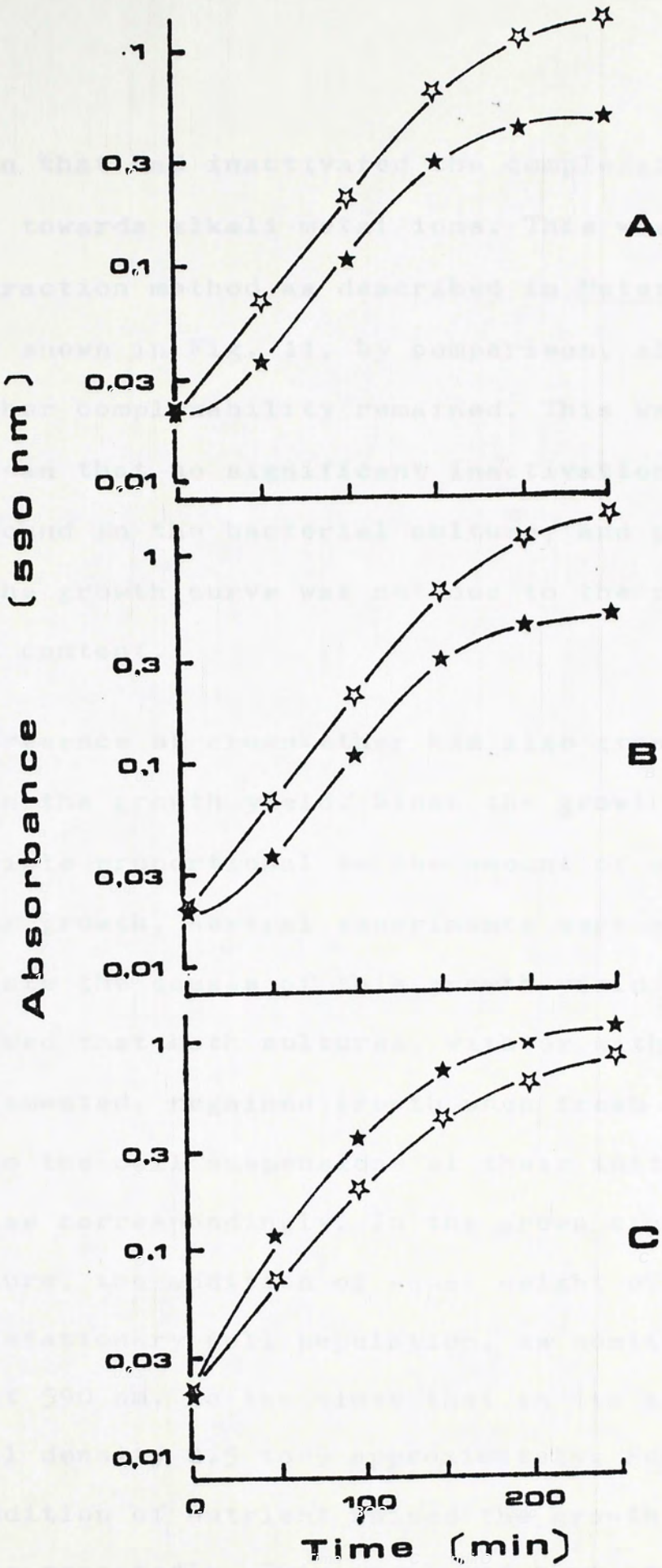
Crown Ether		Viability of Escherichia coli		
Type	Concentration ($x10^{-2}M$)	Colony/Plate	Survival (%)	Inhibition (%)
18-Crown-6	0	144±5	100	0
	0.1	143±7	99.3	0.7
	0.5	138±7	95.8	4.2
	1	131±5	91.0	9.0
Benzo-18-crown-6	0	144±5	100	0
	0.1	123±12	85.4	14.6
	0.5	73±5	50.7	49.3
	1	36±3	25.0	75.0
4'-Methyl-benzo-18-crown-6	0	659±11	100	0
	0.05	646±6	98.0	2.0
	0.25	615±5	93.3	6.7
	0.5	251±3	38.1	61.9
Dicyclohexyl-18-crown-6	0	144±5	100	0
	0.0524	110±9	76.4	23.6
	0.252	74±11	51.4	48.6
	0.524	24±13	16.7	83.3

on growth lag in NaTB cultures (see Table 12).

The survived individuals may be adapted to the toxic environment or being spontaneous resistant mutants to the toxicant. Fig. 10 presented some observation that showed the 'resistant' enriched populations, as selected by continuous culturing in the toxic medium, following by a reculturing in pure growth medium to differentiate its genetic change against other possible adaptation phenomena, however, still retained its characteristic lag in crown ether supplemented growth medium, seemingly no resistance had been developed. However, cultures that had been sufficiently exposed to crown ether and was immediately transfered to plain growth medium at their logarithmic growth state, a slight increase in growth rate was detected. It appeared that the bacterial population, after exposure to crown ether, had been adapted to the adverse environment, possibly through rearrangement of cellular metabolism. And in the sudden release of the repression, an even better growth was expressed.

Crown ether, being lipid soluble, was expected to have been incorporated into bacterial membrane. Aside from this loss, a determination of the residual crown ether content in the exhausted culture medium was done to see whether there was any substantiative biodegradation or

Figure 10. Effect of Crown Ether on the Growth Curve of E. coli that Had Been Precultured in Various Conditions. (☆) Growth in trytone broth control and (★) growth in 5×10^{-3} M dicyclohexyl-18-crown-6. Panel A : Bacteria employed had never been exposed to crown ether before. Panel B : Bacterial cells inoculated were precultured once, approximately for 5 generations, in 5×10^{-3} M dicyclohexyl-18-crown-6 supplemented medium, and followed by reculturing once in pure tryptone medium to minimize possible crown ether resistant effect. Panel C : Bacteria inoculated had been cultured for more than 10 generations in 5×10^{-3} M dicyclohexyl-18-crown-6 supplemented growth medium.


Absorbance (590 nm)
1
0,3
0,1
0,03
0,01
A
B
C
0
100
200
Time (min)

modification that had inactivated the complexability of crown ether towards alkali metal ions. This was done by solvent extraction method as described in Materials & Methods. As shown in Fig. 11, by comparison, about 95 % of crown ether complexability remained. This was interpreted to mean that no significant inactivation of crown ether was found in the bacterial culture, and postlag growth in the growth curve was not due to the reduction in toxicant content.

The presence of crown ether had also created a reduction in the growth yield. Since the growth yield is in principle proportional to the amount of nutrient utilized for growth, several experiments were attempted to investigate the causes of this growth yield reduction. Fig. 12 showed that both cultures, with or without crown ether supplemented, regained growth when fresh nutrient was added to the cell suspensions at their initial stationary phase correspondingly. In the crown ether supplemented culture, the addition of equal weight of nutrient raised the stationary cell population, as monitored by turbidity at 590 nm, to two times that in its absence, from optical density 2.5 to 5 approximately. Furthermore, a second addition of nutrient raised the growth yield to higher value repeatedly. Th[illegible]ed that at stationary

Figure 11. 18-Crown-6 Complexability in Freshly Prepared and Exhausted Crown Ether Supplemented Tryptone Broth. Complexability was studied by the solvent extraction method as described in Materials and Methods. Potassium picrate was chosen as the complexed template. The original 18-crown-6 concentration was 10^{-2} M. Absorbance of crown ether-potassium picrate detected in (—) fresh and (----) exhausted crown ether supplemented growth medium.

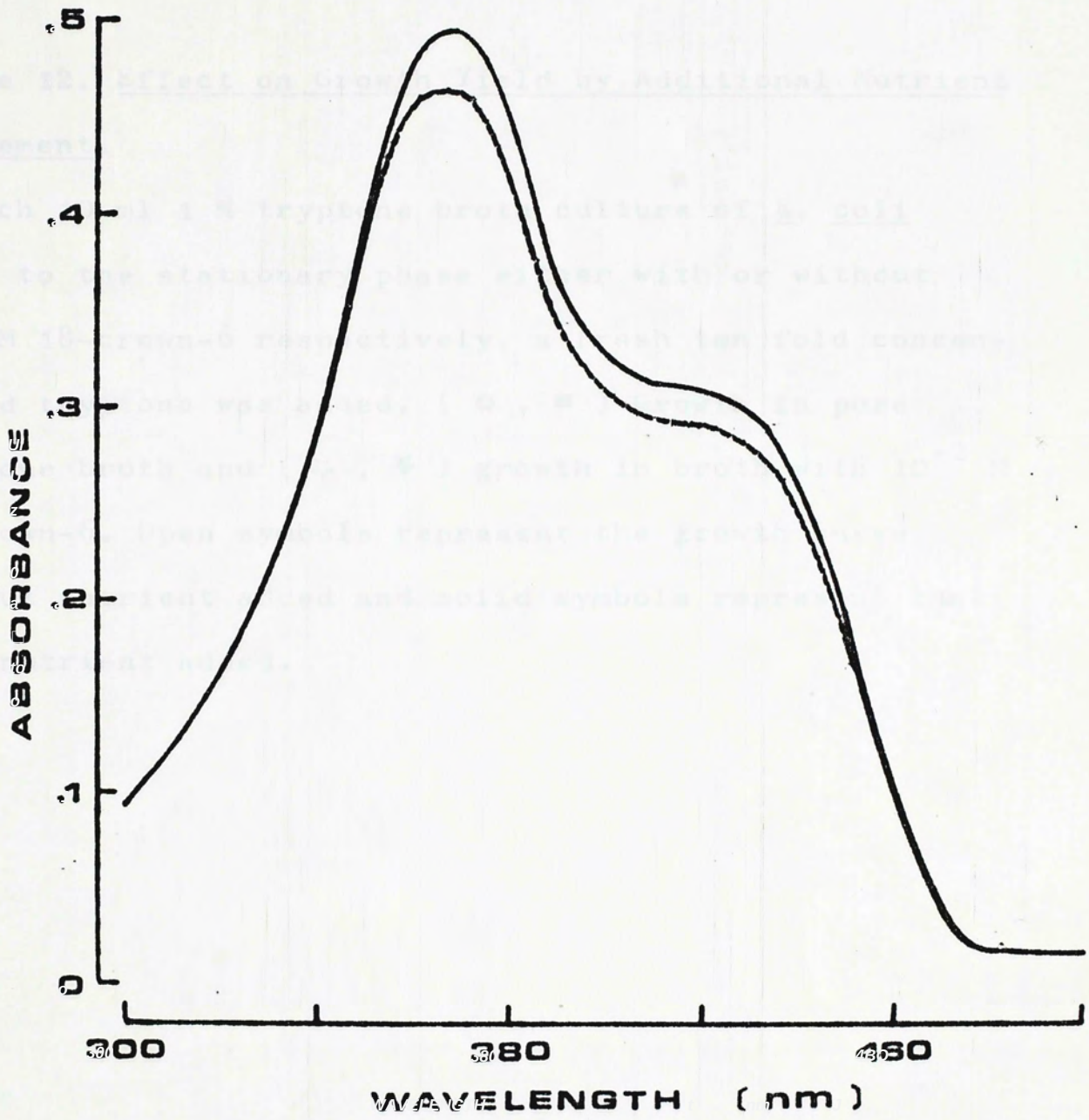
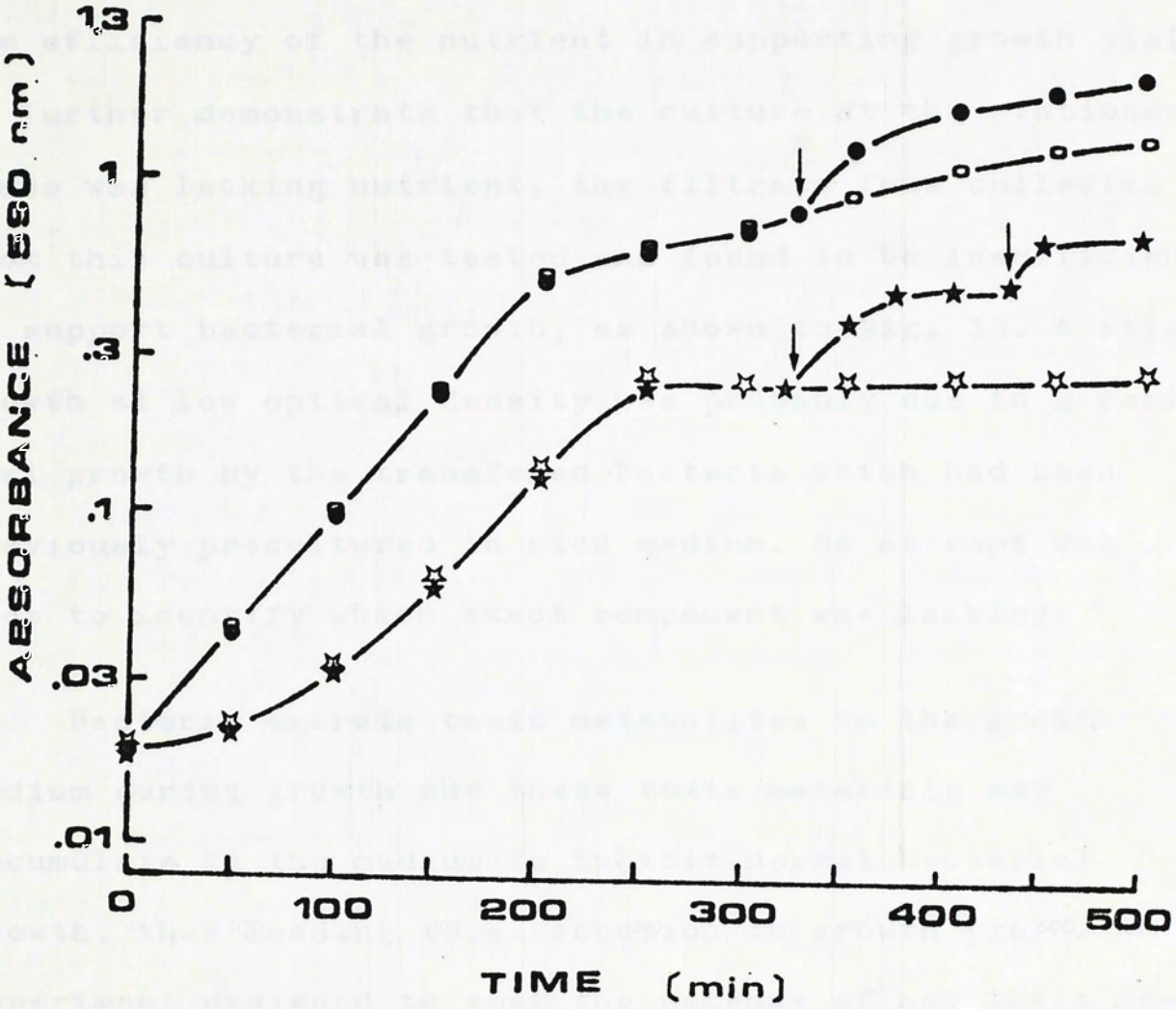

ABSORBANCE
0
1
2
3
4
5
300
380
480
WAVELENGTH (nm)

Figure 12. Effect on Growth Yield by Additional Nutrient Supplement.

To each 10 ml 1 % tryptone broth culture of E. coli grown to the stationary phase either with or without 10^{-2} M 18-crown-6 respectively, a fresh ten fold concentrated tryptone was added. (o , ●) Growth in pure tryptone broth and (☆ , ★) growth in broth with 10^{-2} M 18-crown-6. Open symbols represent the growth curve without nutrient added and solid symbols represent that with nutrient added.


ABSORBANCE (590 nm)
1.3
1
.3
.1
.03
.01
0
100
200
300
400
500
TIME (min)

phase, the growth yield was limited because of nutrient shortage and that uninterupted growth was ever ready on receiving a new nutrient supplement. This was equivalent to suggesting that the presence of crown ether lowered the efficiency of the nutrient in supporting growth yield. To further demonstrate that the culture at the stationary phase was lacking nutrient, the filtrate thus collected from this culture was tested and found to be insufficient to support bacterial growth, as shown in Fig. 13. A slight growth at low optical density was probably due to a residual growth by the transfered bacteria which had been previously precultured in rich medium. No attempt was made to identify which exact component was lacking.

Bacteria excrete toxic metabolites to the growth medium during growth and these toxic materials may accumulate in the medium to inhibit normal bacterial growth, thus leading to a reduction in growth yield. An experiment designed to test the potency of any toxic metabolite was performed by mixing the exhausted with the fresh crown ether supplemented tryptone broth in various ratios and studied the growth yield and growth curve pattern of E. coli in each of these mixed media. It was found however, as depicted in Fig. 14, that up to 60 % exhausted broth, no significant inhibition was

Figure 13. Growth Curves of E. coli in Growth Media Containing of Various Chemical Compositons

(O) Growth in Control tryptone broth, (●) growth in 5×10^{-3} M 4'-methyl-benzo-18-crown-6 supplemented tryptone broth and (▲) growth in exhausted crown ether supplemented tryptone broth medium. The growth exhausted crown ether supplemented tryptone broth was collected as sterile filtrate by passing the solution mixture of a E. coli culture grown in 5×10^{-3} M 4'-methyl-banzo-18-crown-6 supplemented tryptone broth to its early stationary phase through a sterile 0.45 um pore size membrane filter.

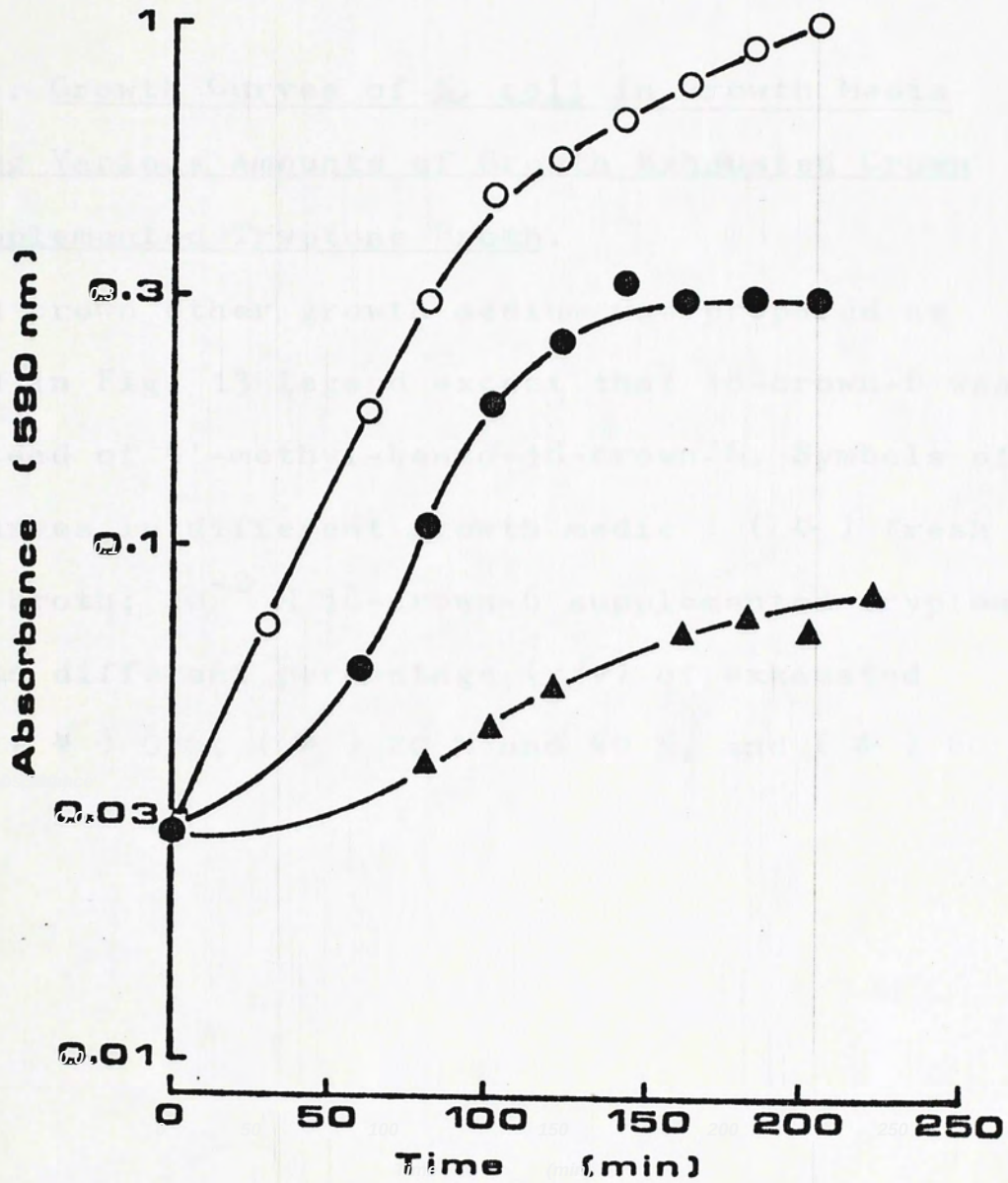
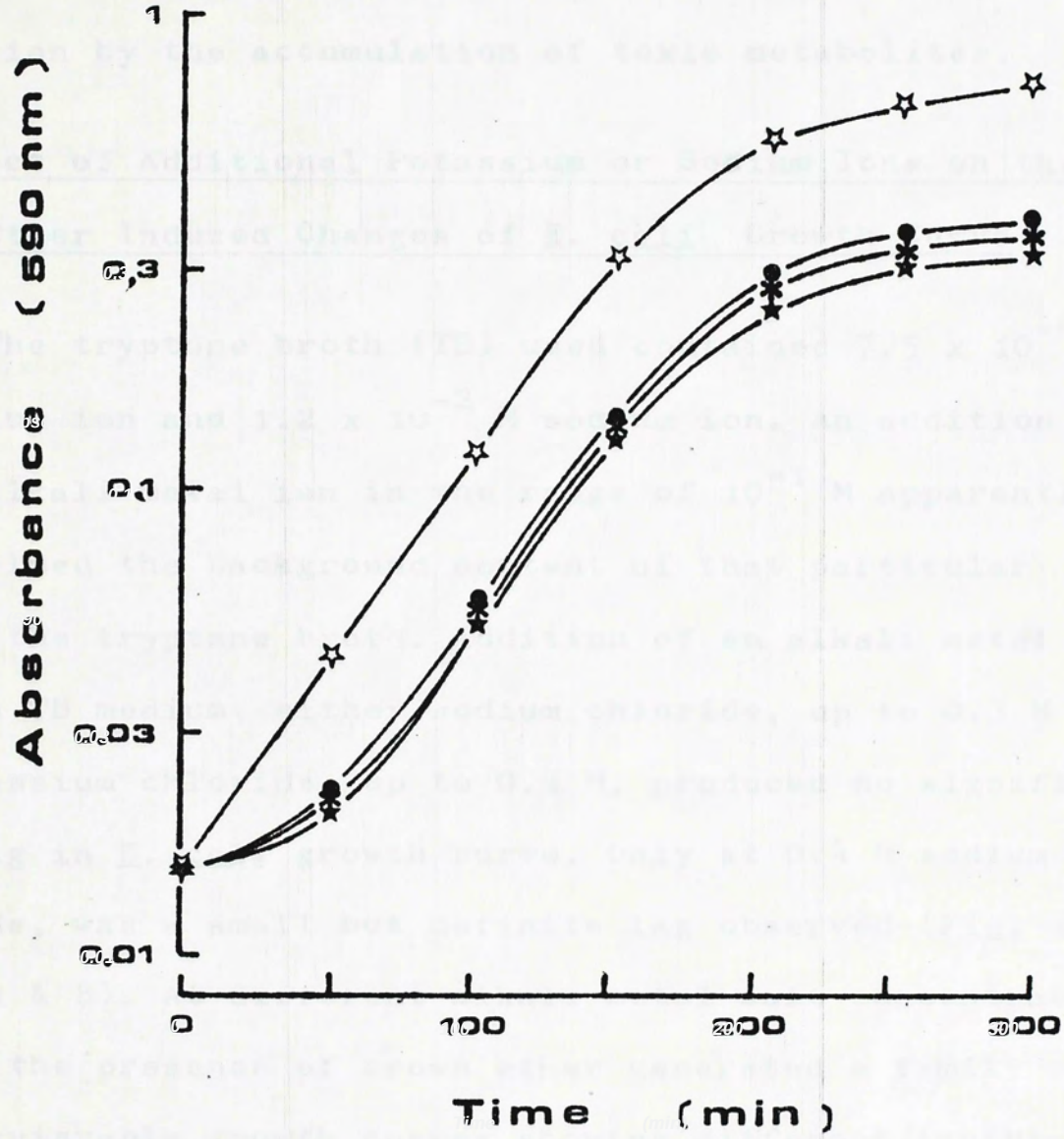

1
0.3
0.1
0.03
0.01
Absorbance (590 nm)
0
50
100
150
200
250
Time (min)

Figure 14. Growth Curves of *E. coli* in Growth Media Containing Various Amounts of Growth Exhausted Crown Ether Supplemented Tryptone Broth.

Exhausted crown ether growth medium was prepared as described in Fig. 13 legend except that 18-crown-6 was used instead of 4'-methyl-benzo-18-crown-6. Symbols of growth curves in different growth media : (✡) fresh tryptone broth; 10^{-2} M 18-crown-6 supplemented tryptone broth with different percentage (v/v) of exhausted medium : (✦) 0 %, (●) 20 % and 40 %, and (*) 60 %.


Absorbance (590 nm)
1
0,3
0,1
0,03
0,01
0
100
200
300
Time (min)

observed. This result, taken together with the previous observations, apparently confirmed that a reduction in growth yield by the presence of crown ether was due mainly to a lower growth efficiency rather than a growth inhibition by the accumulation of toxic metabolites.

7. Effect of Additional Potassium or Sodium Ions on the Crown Ether Induced Changes of E. coli Growth Curve

The tryptone broth (TB) used contained 7.5×10^{-4} M potassium ion and 1.2×10^{-2} M sodium ion. An addition of an alkali metal ion in the range of 10^{-1} M apparently overwhelmed the background content of that particular ion in the tryptone broth. Addition of an alkali metal salt in TB medium, either sodium chloride, up to 0.3 M or potassium chloride, up to 0.4 M, produced no significant lag in E. coli growth curve. Only at 0.4 M sodium chloride, was a small but definite lag observed (Fig. 15, panel A & B). At different alkali metal salt concentrations, the presence of crown ether generated a family of distinguishable growth curves showing different inhibition patterns (Fig. 15, panel C to H). In these salt supplemented growth curves, the change in the lag was more regular and could be conveniently measured. Fig. 16 reported the lag shift which was the effect due to the crown ether at various sod[illegible]ride (the lag at 0.4 M

Figure 15. Effect of Additional Potassium or Sodium Ions on the Crown Ether Induced Changes of E. coli Growth Curve

E. coli Growth curves in tryptone broth supplemented with either sodium chloride (panels A,C,E,G) or potassium chloride (panels B,D,F,H) were shown. Panels A and B (control with no crown ether), showed the growth curves at various additional salt concentrations : (O) Tryptone alone, (■) 0.1 M/ 0.2 M were indistinguisable, (✶) 0.3 M and (✱) 0.4 M salt. In panels C to H, a crown ether at a concentration of 5×10^{-3} M was added : 18-crown-6 (panels C,D), dicyclohexel-18-crown-6 (panels E,F) and 4'-methyl-benzo-18-crown-6 (panels G,H). The growth curves at various additional salt concentration were shown : (O) Tryptone broth alone, (●) crown ether, and crown ether plus (▲) 0.1 M, (■) 0.2 M, (✶) 0.3 M and (✱) 0.4 M.

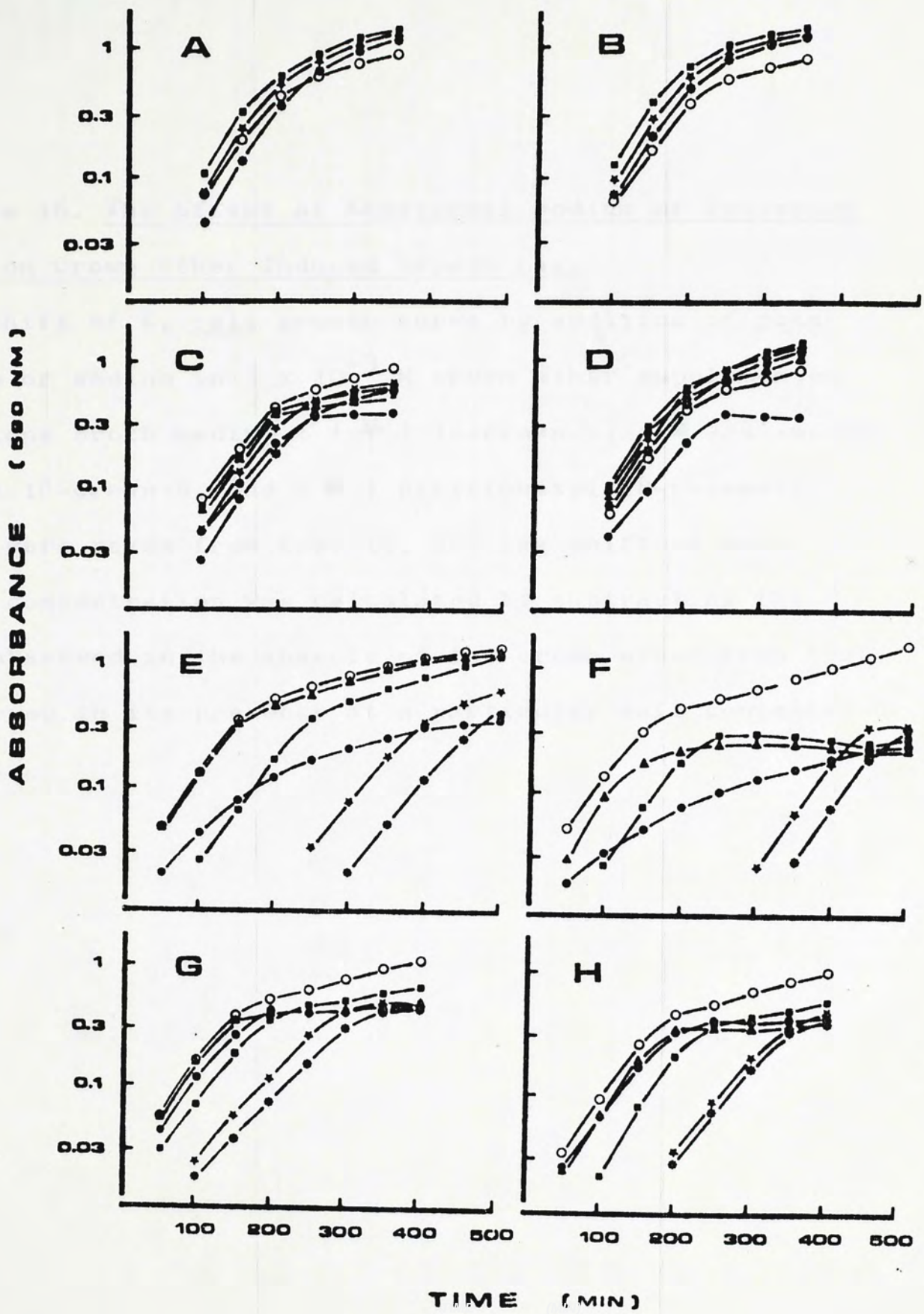
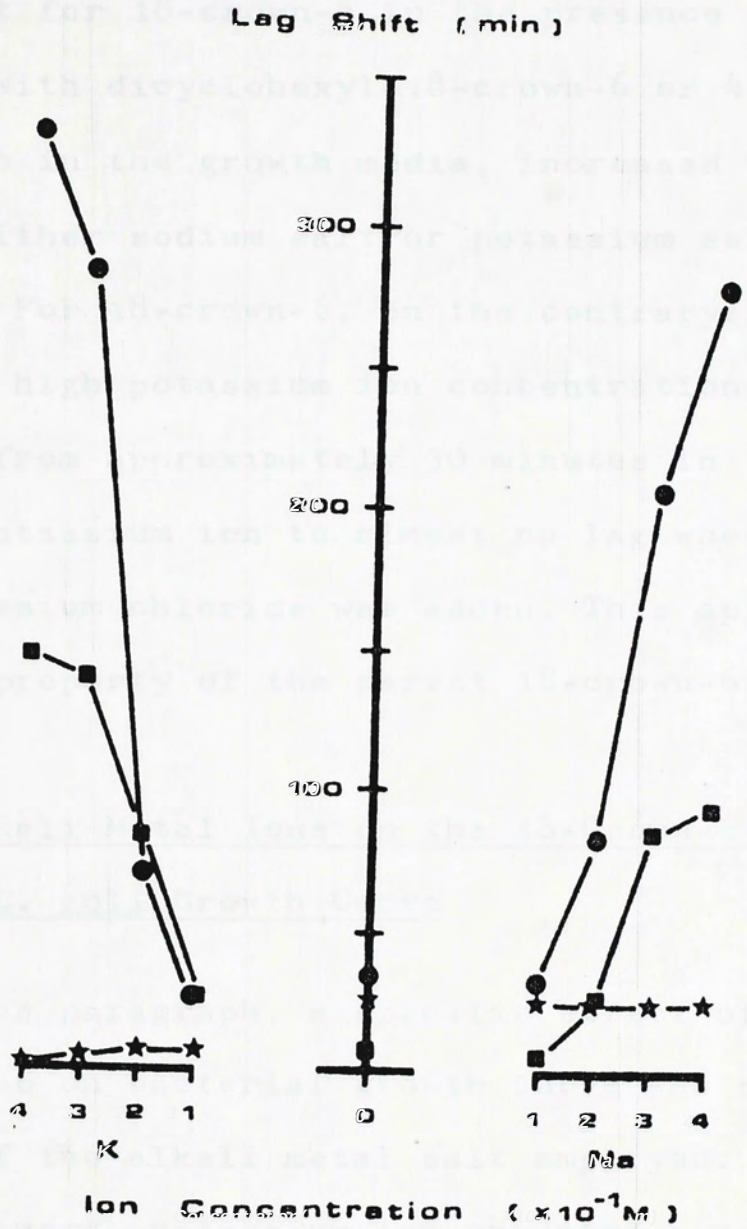

A
B
C
D
E
F
G
H
1
0.3
0.1
0.03
ABSORBANCE (660 NM)
100
200
300
400
500
TIME (MIN)

Figure 16. The Effect of Additional Sodium or Potassium Ions on Crown Ether Induced Growth Lag.

Lag shift of E. coli growth curve by addition of potassium or sodium in 5×10^{-3} M crown ether supplemented tryptone broth medium : (★) 18-crown-6, (■) 4'-methylbenzo-18-crown-6, and (●) dicyclohexyl-18-crown-6. Data were taken from Fig. 15, the lag shift at each salt concentration was calculated by subtracting the lag observed in the absence of the crown ether from that observed in its presence at a particular salt concentration.


Lag Shift (min)
300
200
100
4 3 2 1
K
0
1 2 3 4
Na
Ion Concentration (x10^{-1}M)

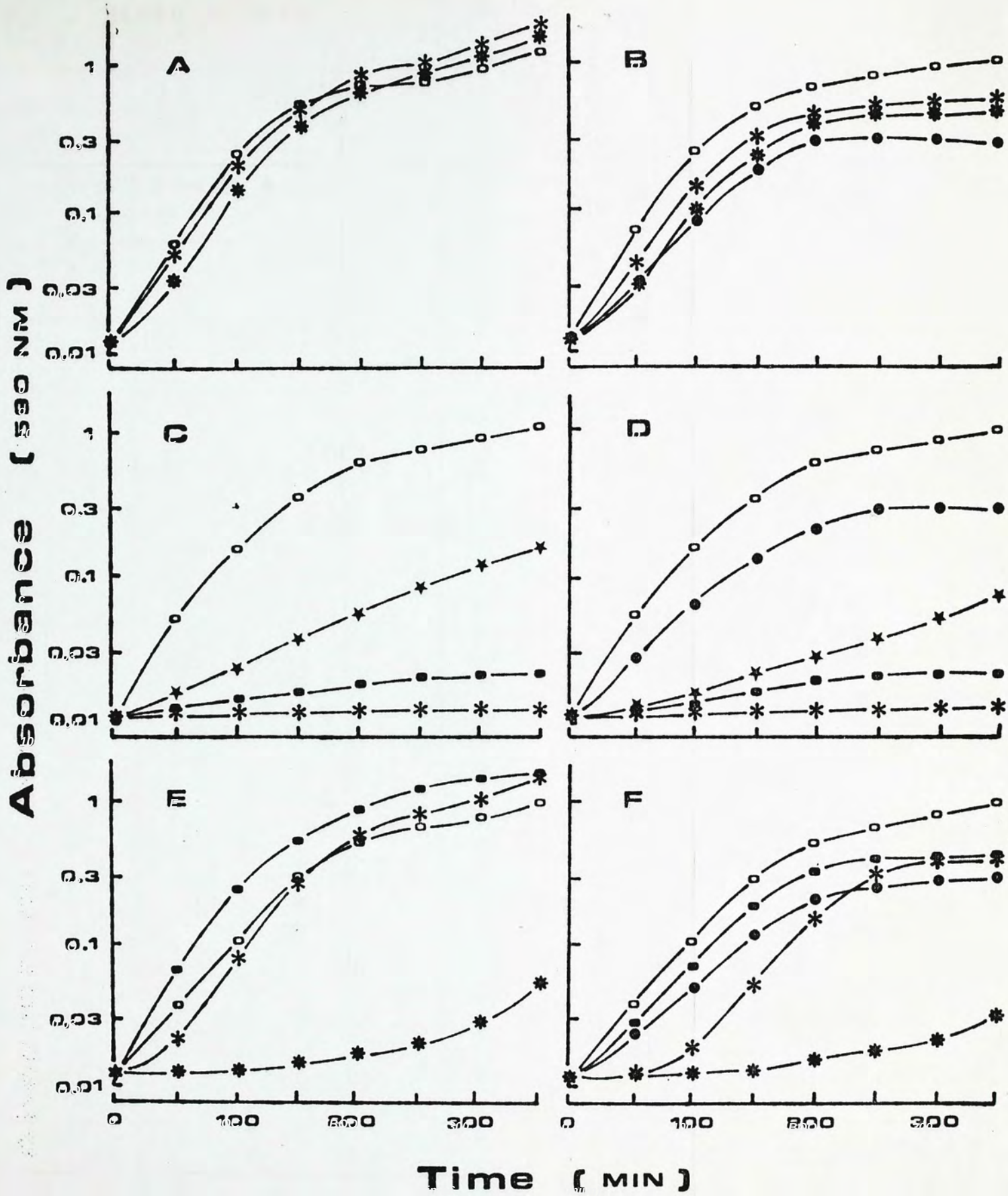
NaCl was subtracted from the gross effect) or potassium chloride concentrations. It was apparent that the lag shift, was affected by the concentration of the alkali metal ion except for 18-crown-6 in the presence of sodium chloride. With dicyclohexyl-18-crown-6 or 4'-methylbenzo-18-crown-6 in the growth media, increased the concentration of either sodium salt or potassium salt lengthened the lag. For 18-crown-6, on the contrary, a rescue was observed at high potassium ion concentrations, shortening the lag from approximately 30 minutes in the absence of additional potassium ion to almost no lag when 4×10^{-1} M potassium chloride was added. This appeared to be a unique property of the parent 18-crown-6.

8. Effect of Alkali Metal Ions on the 18-Crown-6 Induced Changes of the *E. coli* Growth Curve

In previous paragraph, a specific effect of the parent 18-crown-6 on bacterial growth curve was produced by the nature of the alkali metal salt employed. While sodium ion was inert, potassium ion produced a rescuing effect. A similar study was exerted to cover the other group IA alkali metals. The results as demonstrated in Fig. 17, showed that rubidium behaved very much like potassium ion. Lithium and cesium, being themselves

Figure 17. Effect of Rubidium, Cesium and Lithium Ions on *E. coli* Growth Curve in the Presence of 10^{-2} M 18-Crown-6.

Growth curves in tryptone broth supplemented with RbCl (panels A,B), CsCl (panels C,D) and LiCl (panels E,F) were shown. Panels A, C and E are the controls without crown ether added. Panels B, D and F, are tryptone broth supplemented with 10^{-2} M 18-crown-6. Without additional ions, (○) Tryptone broth and (●) tryptone supplemented with 18-crown-6. In addition of ions in growth medium, in panels A and B, Rb at (✱) 0.1 M, 0.2 and 0.3 M, and (✱) 0.4 M. In panel C and D, Cs at (✦) 0.1 M, (●) 0.2 M and (✱) 0.3 and 0.4 M. In panels E and F, Li at (○) 0.1 M and 0.2 M, (✱) 0.3 M and (✱) 0.4 M.


A
B
C
D
E
F
1
0.3
0.1
0.03
0.01
0
100
200
300
Absorbance (560 NM)
Time (MIN)

toxic at high concentrations, showed an irregular growth curve pattern. This study further indicated the resemblance of rubidium to potassium in bacterial growth physiology. In fact, rubidium had been reported to support bacterial growth as well as potassium (MacLeod & Snell, 1948; Lester, 1958).

9. Effect of 18-Crown-6 on ^{86}Rb Transport Through E. coli Cell Membrane

E. coli cells when not supplied with nutrient were almost incapable of accumalating ^{86}Rb, but addition of glucose abruptly induced a potent ^{86}Rb uptake by the bacterial cells (Fig. 18). 18-Crown-6 at concentration of 10^{-2} M, only slightly reduced the ^{86}Rb uptake mobilized by bacterial active transport system, and showed simply no effect on nutrient limited bacterial cells.

However, the ion retention property of ^{86}Rb loaded cells was reduced by the addition of 18-crown-6 (Fig. 19). The loss of ^{86}Rb radioactivity was concentration dependent and was more significant for longer period (Fig. 20). High dosage of 18-crown-6 (5×10^{-2} M to 1×10^{-1} M), as well as 8 µg/ml valinomycin, induced rapid loss of ^{86}Rb within 10 minutes, while lower dosage required longer period for more significant reduction of radio-

Figure 18. Effect of 18-Crown-6 on E. coli ^{86}Rb Uptake

Freshly logarithmic phase of E. coli cells were harvested, washed and resuspended in 25 mM sodium maleate buffer containing 10 mM ^{86}RbCl with addition of (✡) no other chemicals, (✦) 10^{-2} M 18-crown-6, (O) 0.1 M glucose, (●) 0.1 M glucose and 10^{-2} M 18-crown-6. At appropriate intervals, a 1-ml portion of the bacterial cells were filtered through millipore membrane and washed with five 1-ml 2 mM $MgCl_2$, as described in Materials and Methods.

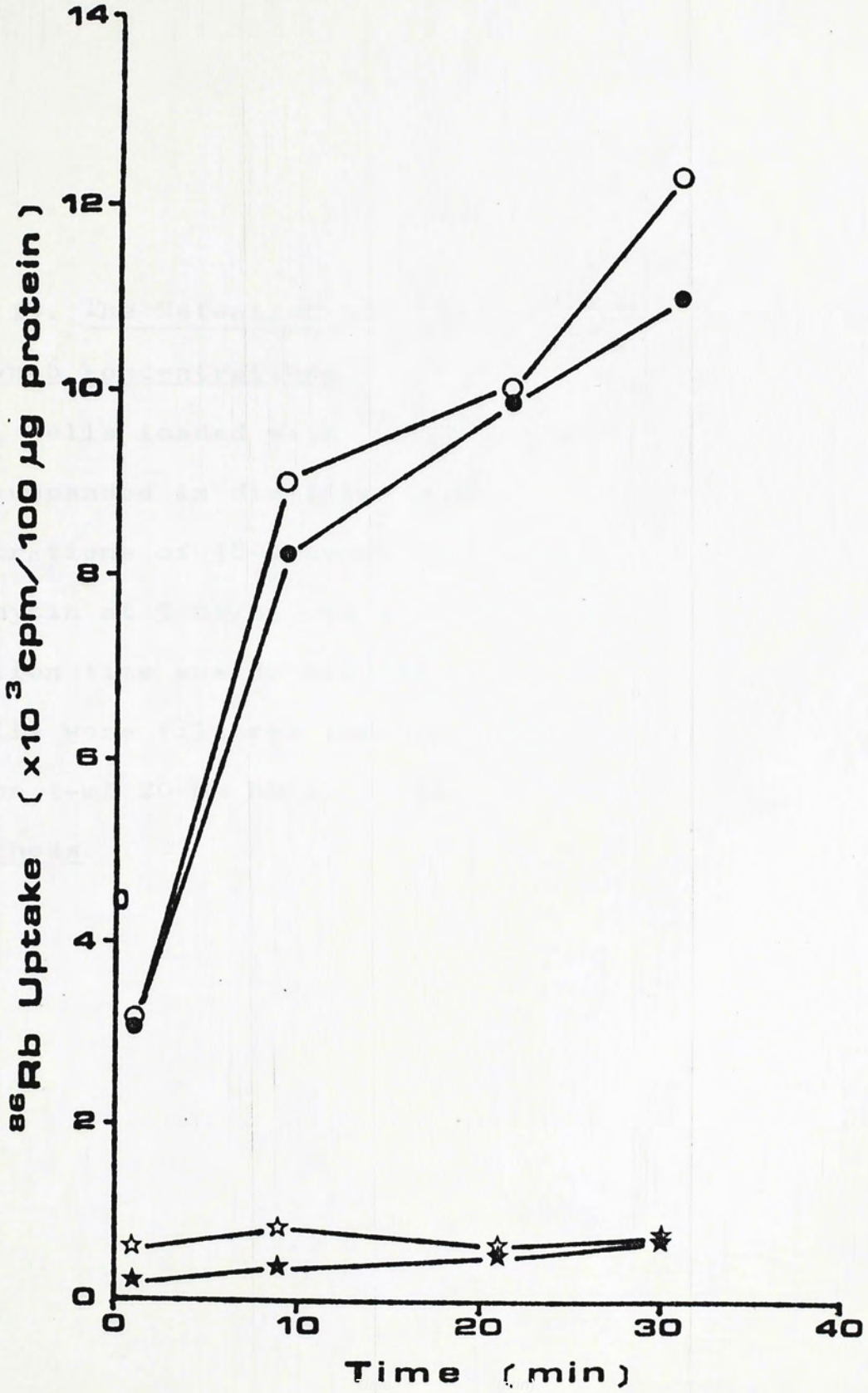

14
12
10
8
6
4
2
0
^{86}Rb Uptake (x10^{3} cpm/ 100 µg protein)
0
10
20
30
40
Time (min)

Figure 19. The Retention of ^{86}Rb in E. coli at Various 18-Crown-6 Concentrations.

E. coli cells loaded with ^{86}Rb were harvested, washed and resuspended in distilled water, in which various concentrations of 18-crown-6 were added. An antibiotic valinomycin at 5 ug/ml was also included (✡). The incubation time was 30 minutes, at 37 C water bath, and the cells were filtered through millipore membrane, washed with ten 1-ml 20 mM RbCl, as described in Materials and Methods.

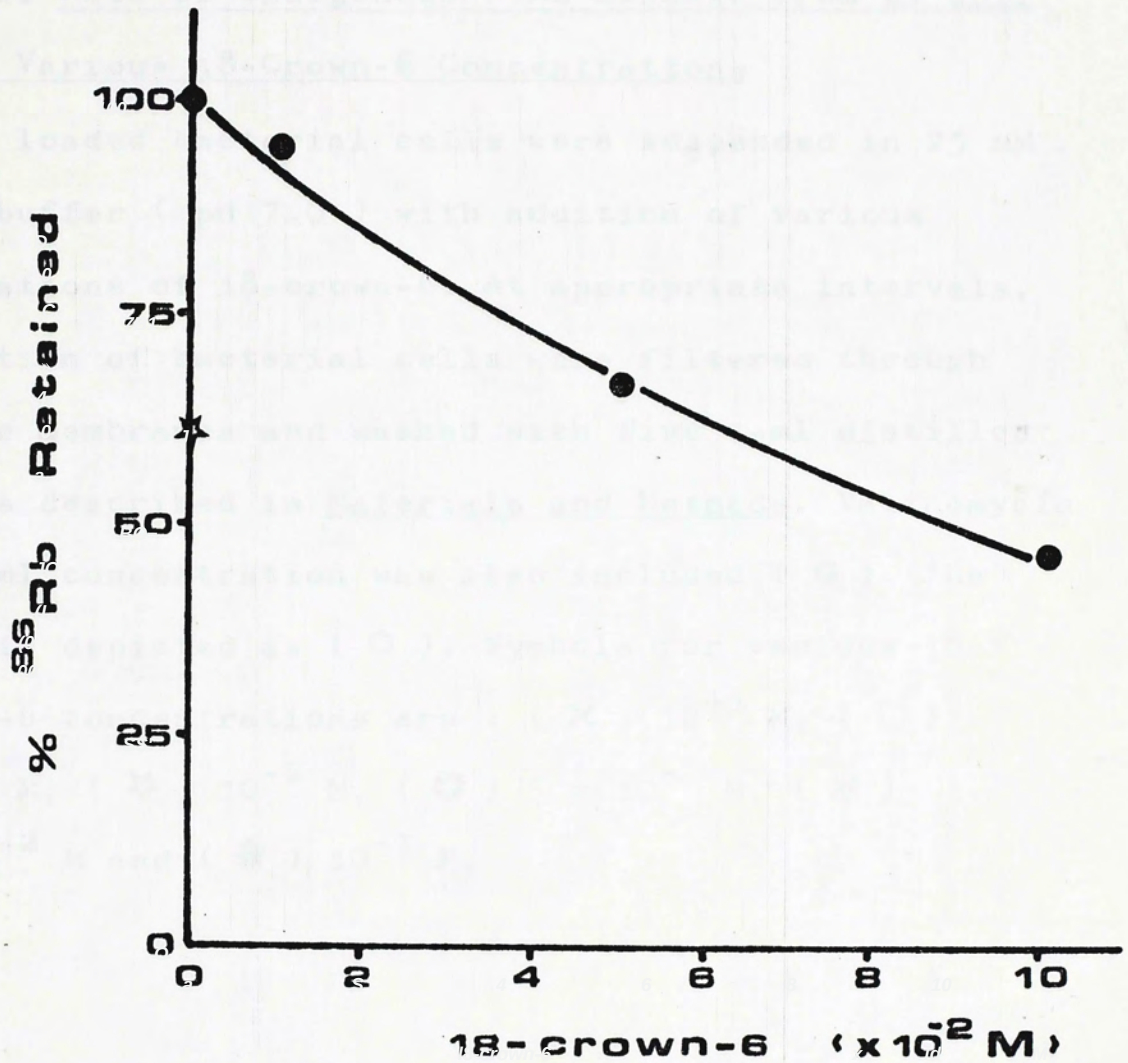

100
75
50
25
0
% ^{86}Rb Retained
0
2
4
6
8
10
18-crown-6 (x 10^{-2} M)

Figure 20. Rate of Endogenous ^{86}Rb Leakage from E. coli Cells at Various 18-Crown-6 Concentrations

The ^{86}Rb loaded bacterial cells were suspended in 25 mM maleate buffer (pH 7.0) with addition of various concentrations of 18-crown-6. At appropriate intervals, 1-ml portion of bacterial cells were filtered through millipore membranes and washed with five 1-ml distilled water, as described in Materials and Methods. Valinomycin at 8 ug/ml concentration was also included (●). The control is depicted as (O). Symbols for various 18-18-crown-6 concentrations are : (✕) 10^{-3} M, (□) 5×10^{-3} M, (✰) 10^{-2} M, (✪) 5×10^{-2} M, (✱) 7.5×10^{-2} M and (✱) 10^{-1} M.

^{86}Rb (cpm/100 µg cell protein)
1250
1000
750
500
250
0
0
10
20
30
40
50
60
70
Time (min)

activity. 18-Crown-6 at 10^{-3} M, the lowest concentration studied, appeared to be still effective in enhancing ^{86}Rb permeability through bacterial membrane.

10. Effect of Crown Ether on Bacterial Respiration

Bacterial respiration seemed to be rather sensitive to crown ether. At as low as 10^{-10} M crown ether concentration, a slight reduction in bacterial respiration was observed (Fig. 21). This was followed by slow but gradual further reduction to about 10^{-4} M. Among the four chemicals tested, benzo-18-crown-6 and 4'-methyl-benzo-18-crown-6 showed similar effect while the parent 18-crown-6 and dicyclohexyl-18-crown-6 exhibited less potency. However, at crown ether concentration over 10^{-4} M, a sharp decrease in bacterial respiration rate was detected. This was most severe in dicyclohexyl-18-crown-6 and the parent 18-crown-6 appeared to be the least toxic one. Essentially, repiration was reduced to undetectable slow rate at 10^{-2} M of the crown ethers except the parent 18-crown-6, which although had initiated a similar rapid reduction in bacterial respiration, a comparatively high rate was still retained at 10^{-2} M of the chemical.

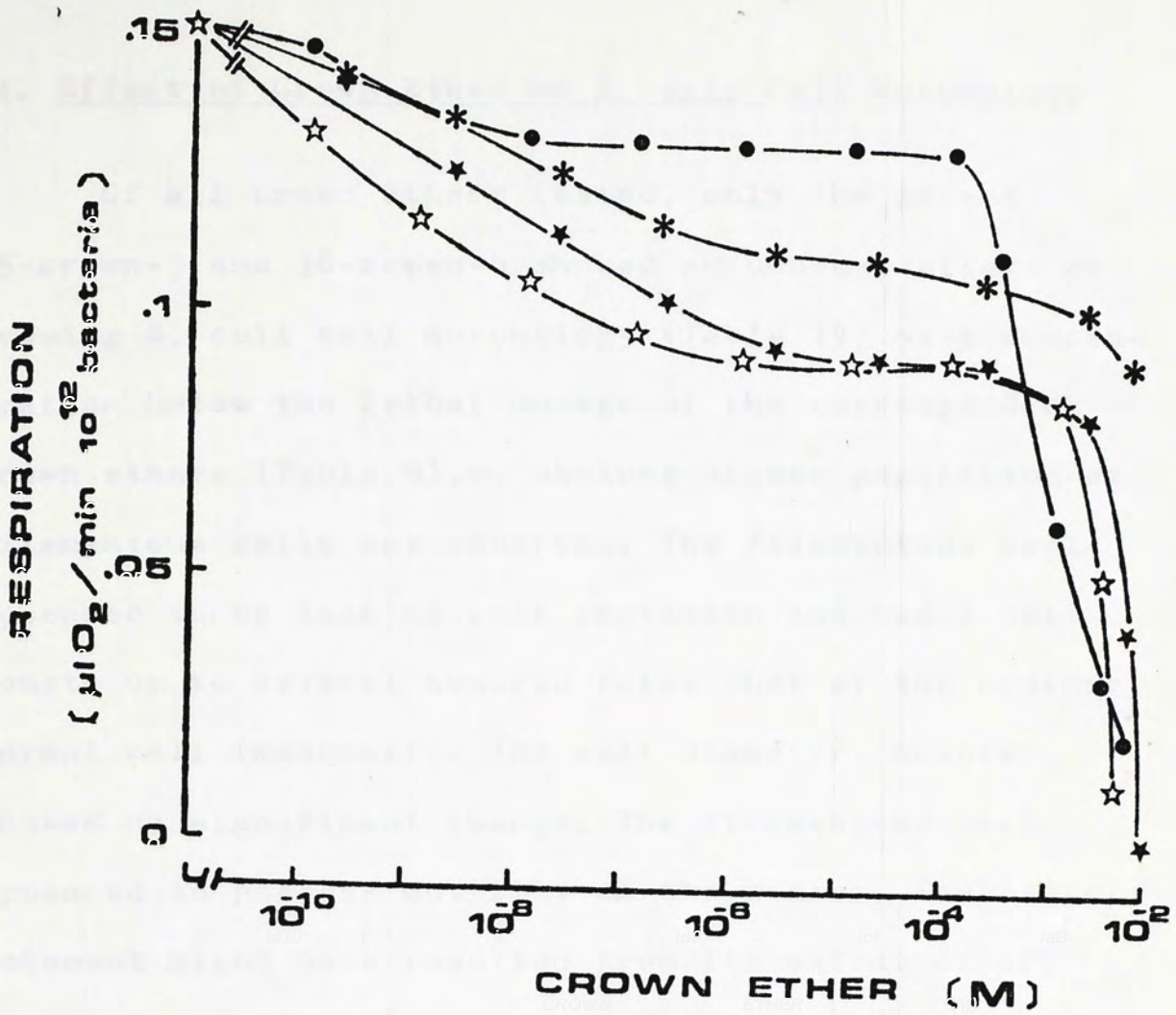


Figure 21. Effect of Crown Ether on Bacterial Respiration. The respiration rate of E. coli was measured amd calculated as described in Materials and Methods. Repiration rates affected by corresponding crown ether are presented as : (*) 18-crown-6, (★) benzo-18-crown-6, (☆) 4'-methyl-benzo-18-crown-6 and (●) dicyclohexyl-18-crown-6.

11. Effect of Crown Ether on E. coli Cell Morphology

Of all crown ethers tested, only the parent 15-crown-5 and 18-crown-6 showed an unusual effect on growing E. coli cell morphology (Table 14) at a concentration below the lethal dosage of the corresponding crown ethers (Table 9).An obvious higher population of filamentous cells was observed. The filamentous cell appeared to be lack of cell septation and had a cell length up to several hundred folds that of the ordinary normal cell (monocell). The cell diameter, however, showed no significant change. The filamentous cell appeared to possess motility in the medium. The sluggish movement might have resulted from its extraordinary body length. From the growth curve determination as reported in earlier paragraphs, it was apparent that except a possible defect in cell division or septation, this filamentous cell had essentially normal growth rate.

12. Susceptibility of Various Bacterial Species to 18-Crown-6 Induced Filamentous Cell Formation

Among a variety of bacteria studied, filamentous cells were found only in E. coli, Salmonella typhimurium and Bacillus (Table 15). None of the coccus-shape

Table 14. Specificity of Crown Ether in Escherichia coli Filamentous Cell Formation

Crown Ether	Filamentous Cell Formation*	Effective Concentration (M)
15-Crown-5	+	$2.6 \times 10^{-2} - 1.5 \times 10^{-1}$
18-Crown-6	+	$5.0 \times 10^{-3} - 2.5 \times 10^{-2}$
Benzo-18-crown-6	-	N.D.[a]
4'-Methyl-benzo-18-crown-6	-	N.D.
Dicyclohexyl-18-crown-6	-	N.D.

*A negative sign indicates absence of filamentous cell formation in the higher portions of the sublevel concentration range tested (usually starts from 10^{-4} M).

[a]N.D. represents not detected.

Table 15. Effect of 18-Crown-6 on Filamentous Bacterial Cell Formation

Description of Bacterium			Filamentous Cell Formation	Effective 18-Crown-6 Concentration* (M)
Strain	Gram	Shape		
Escherichia coli	-	rod	+	5×10^{-3} - 2.5×10^{-2}
Salmonella typhimurium	-	rod	+	4×10^{-3} - 1.9×10^{-2}
Streptococcus	+	coccus	-	N.D.
Staphylococcus coagulate(+)	+	coccus	-	N.D.
Corynebacterium	+	pleomorphic rod	-	N.D.
Bacillus	+	rod	+	2×10^{-3} - 9×10^{-3}
Halobacterium cutiritium	-	rod	-[a]	N.D.
Staphylococcus coagulate(-)	+	coccus	-	N.D.

*The concentration of 18-Crown-6 studied ranged from 10^{-4}M to 10^{-1}M. The test was done with a 2-fold serial dilution.

[a]Halobacterium cells grown at 10^{-3}M to 4×10^{-3}M 18-Crown-6 swelled to several fold of their original size and appeared transparent.

bacteria showed any variation in their shape or their aggregation structures. Corynebacterium, a pleomorphic rod shape bacterium, i.e. although rod-shape is sometimes observed, it is very flexible and may change to coccus or other irregular shape (Barksdate, 1970; Bergey, 1957), was found to exist in the form of aggregated units with short axial ratio and to have shown no morphological change induced by 18-crown-6. As a result, only those 'strict' rod-shape bacteria, either gram-negative or gram-positive, were susceptible to crown ether induced filamentous cell formation. Halobacterium, although is classified to be a gram-negative rod-shape bacterium (Bergey, 1957), has a remarkable distinctive habitat, membrane structure (Blaurock & Stoeckenius, 1971), cell protein properties and enzyme systems (Hochstein, 1975; Lanyi, 1974). This halophilic bacterium, however, showed significant cell size enlargement at a definite concentration of 18-crown-6 (Table 15). As the cell volume of Halobacterium is suggested to be regulated by intracellular ion concentrations (Ginzburg & Ginzburg, 1976), an enlargement in cell volume might simply reflect a change of ion concentration in the presence of 18-crown-6. In all filamentous cell forming conditions, it was noted that the effective concentration was always slightly lower than the MIC determined in that bacterial

species (Tables 10 & 15).

13. Morphology of the Crown Ether Induced Filamentous E. coli Cell

Filamentous E. coli cells stained with methylene blue, showed increased cell length up to hundred folds or more. As shown in Fig. 22, a population of this crown ether induced filamentous cell had a very heterogenous cell size distribution. The filamentous cells stained for capsule showed rarely the existance of cell septum (Fig. 23). Furthermore, their cell surface morphology was examined more detailly with scanning electron microscope (SEM). The filamentous cell was smooth throughout the whole length , no indication of thinner sites or furrows were found depicting the presence of septum formation sites (Fig. 24). A nuclear staining, however, showed that the filamentous cell was polynucleated (Fig. 25). This nuclear DNA were regularly distributed , probably at a distance corresponding to the normal unit cell length.

14. Distribution of Filamentous E. coli Cells in Relation to Incubation Time and 18-Crown-6 Concentration

In order to study in more quantitative manner the

Figure 22. Gross Morphology of E. coli Filamentous Cells. Bacterial cells grown in pure tryptone broth (A) and those in tryptone broth supplemented with 10^{-2} M 18-crown-6 (B) were stained with Loeffler's methylene blue as described in Materials and Methods. The size of the objects appear in the picture are 3500 folds amplified.

A

B

Figure 23. Filamentous E. coli Cells with Capsule Stained. Filamentous cells were produced by growing E. coli in 1.5×10^{-2} M 18-crown-6 supplemented tryptone broth. Cells were treated with Hiss's capsule stain. The size of the object appeared in the picture was magnified 7000 folds.

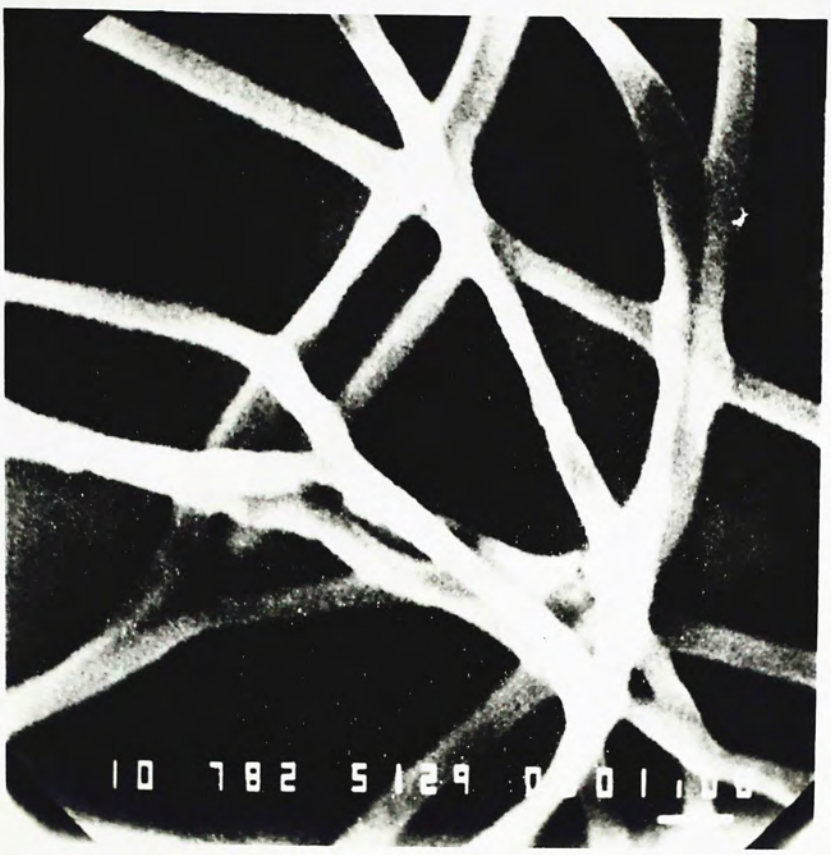


Figure 24. SEM Micrograph of Filamentous E. coli Cells. Sample preparation for SEM studies were as described in Materials and Methods. White line on the lower right hand corner is the amplified 1 μm. Amplification fold of the objects appeared in the photo was magnified 7800 folds.

Figure 25. Filamentous E. coli Cells with Nuclear Structure Stained.

Filamentous cells were treated with Robinow's nuclear stain as described in Materials and Methods. Size of the object appeared in the picture was amplified to 3500 folds.

concentration effect of 18-crown-6 and incubation time on filamentous E. coli cell formation, the individual cell length was measured and their distributions were compared with respect to the percentage of filamentous cell population and the maximum length of the individual cell. The histograms in Fig. 26 represented the cell size distribution at each incubation period. The cell length as well as the percentage of the filamentous cells were shown to be proportional to the period of incubation (Fig. 27). The shape of the curves in Fig. 27 indicated that the increase of filamentous cell length and the population percentage were reaching a somewhat steady state after a culture period of 200 minutes, approximately corresponding to 6 generation times. It was found also that an overnight culture (approximately 16 hours) with the same 1.25×10^{-2} M 18-crown-6 concentration showed essentially similar filamentous cell distribution as that determined at 225 minutes' incubation (Fig. 27 & 30). However, under no condition, at whatever length of incubation period tested, a heterogenous cell size distribution existed.

Within the effective concentration range of 18-crown-6, the percentage and cell length of filamentous cell were concentration dep[illegible]nt ([illegible] 28[illegible]). The

Figure 26. Cell Size Distribution in E. coli Grown for Various Periods in 1.25 x 10^{-2} M 18-crown-6 Supplemented Tryptone Broth.

Cell size distribution at incubation time : (A) 0, (B) 90, (C) 135, (D) 180 and (E) 225 min. The number on the right hand corner refers to the total number of bacteria examined. The normal short cells population percentage are indicated by the values presented on the bars of cell length approaching zero. E. coli normal cell length is suggested to be 2.5 um (Pierucci, 1978).

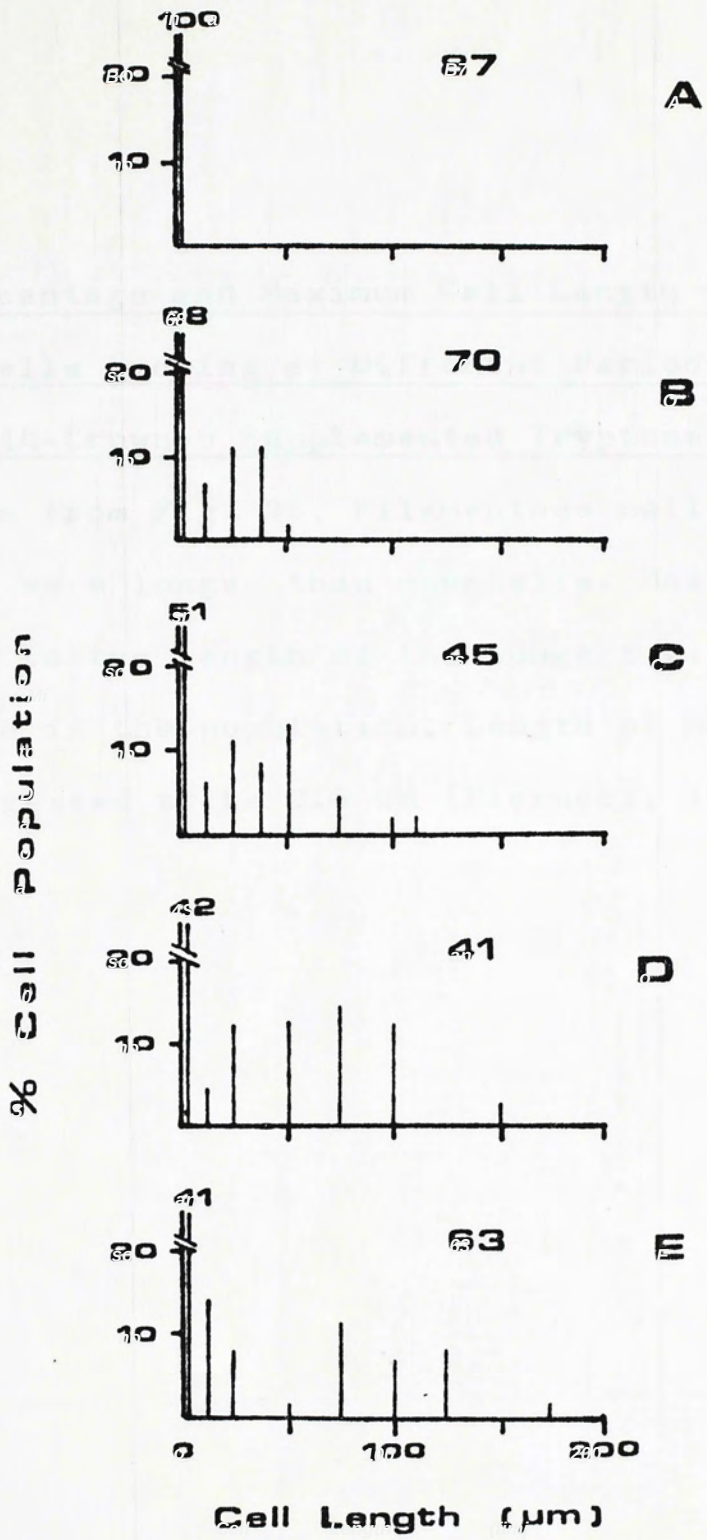

100
80
10
87
A
68
80
10
70
B
51
80
10
45
C
42
80
10
41
D
41
80
10
63
E
0
100
200
% Cell Population
Cell Length (µm)

Figure 27. Percentage and Maximum Cell Length of Filamentous *E. coli* Cells Growing at Different Period in 1.25×10^{-2} M 18-Crown-6 Supplemented Tryptone Broth. Data were taken from Fig. 26. Filamentous cells refered to those which were longer than monocells. Maximum cell length refered to the length of the longest filamentous cell registered in the population. Length of normal *E. coli* is suggested to be 2.5 um (Pierucci, 1978).

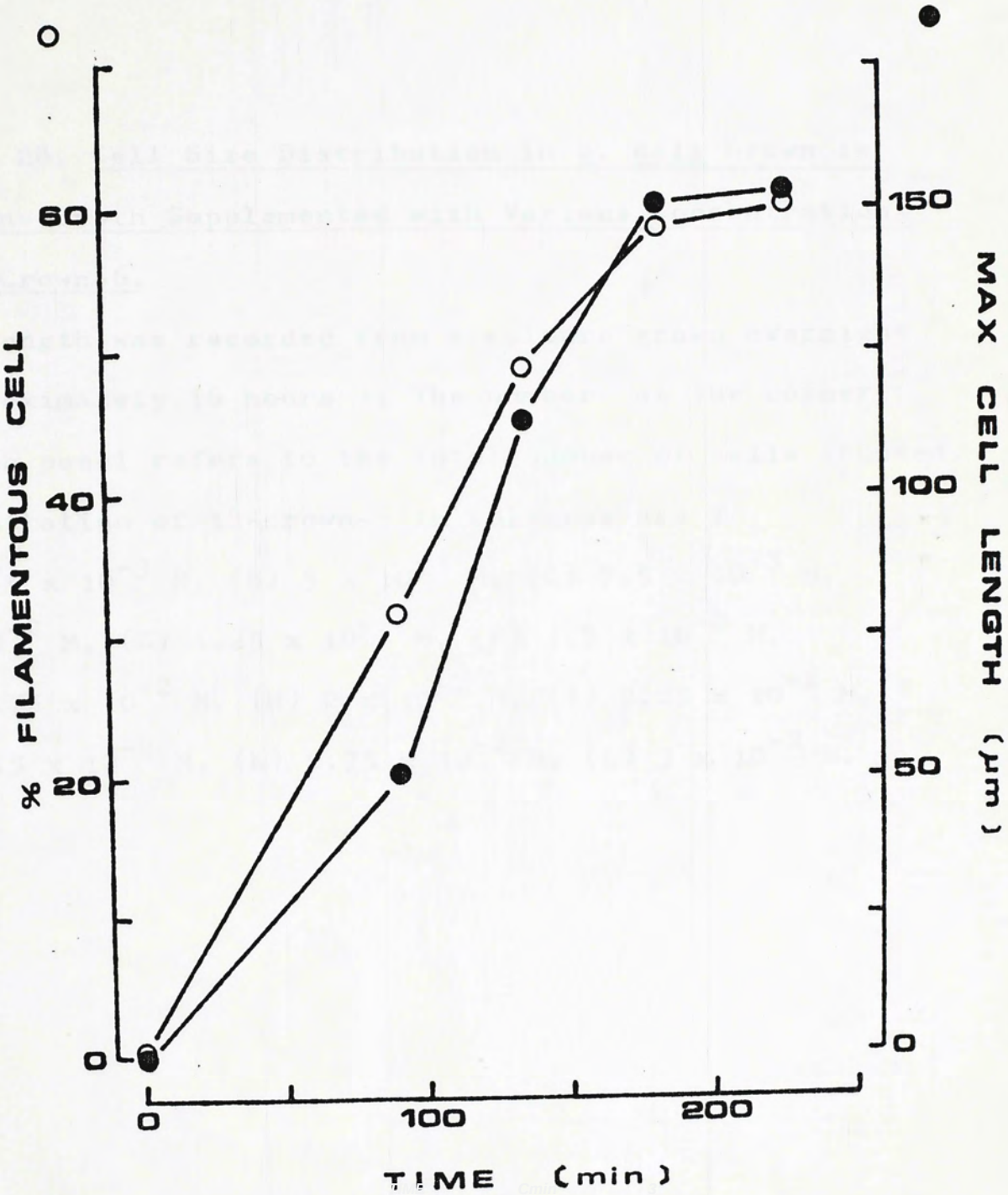

% FILAMENTOUS CELL
0
60
40
20
0
MAX CELL LENGTH (μm)
150
100
50
0
0
100
200
TIME (min)

Figure 28. Cell Size Distribution in E. coli Grown in Tryptone Broth Supplemented with Various Concentrations of 18-Crown-6.

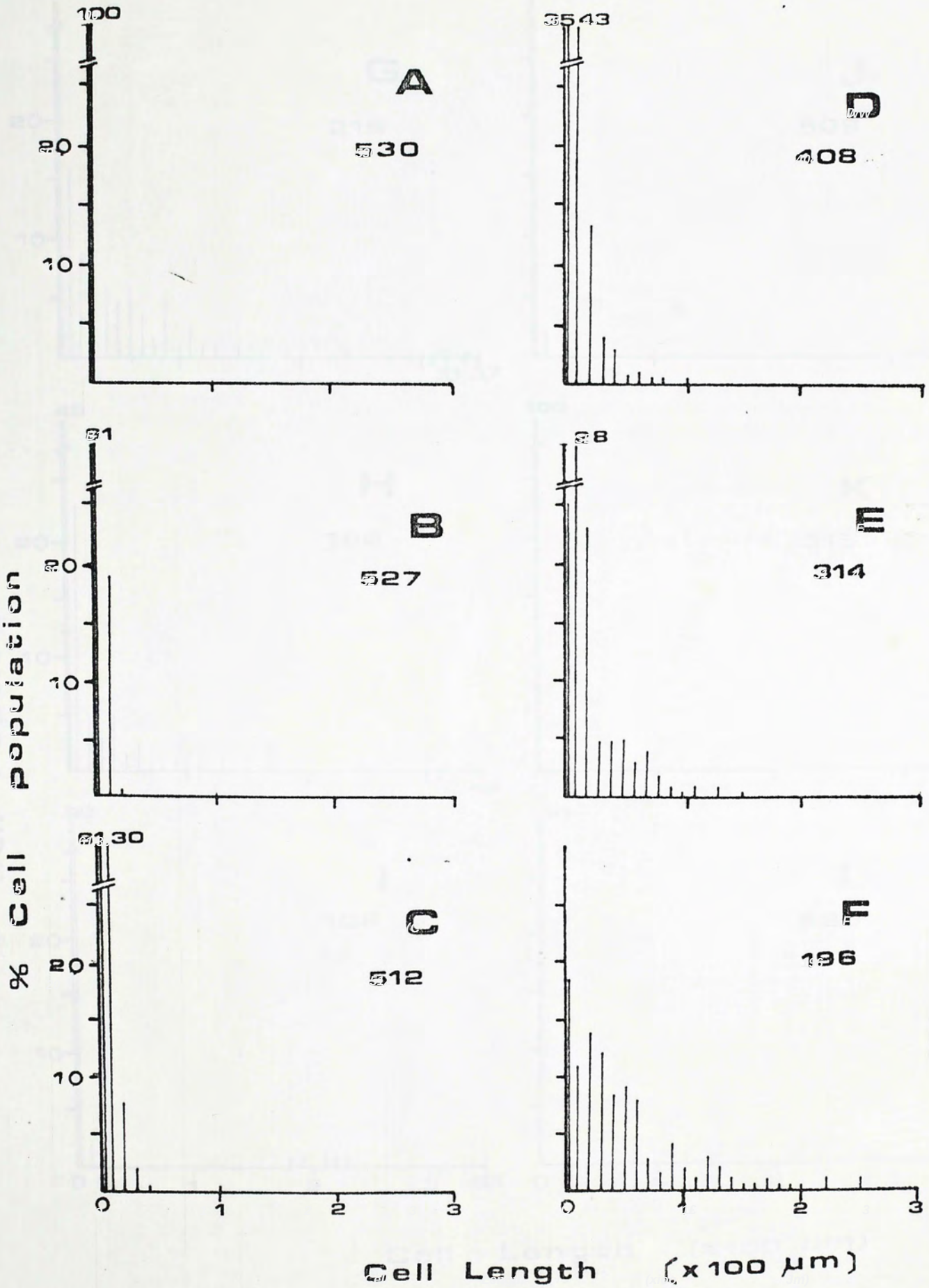
Cell length was recorded from a culture grown overnight (approximately 16 hours). The number at the corner of each panel refers to the total number of cells counted. Concentration of 18-crown-6 in cultures are :

(A) 2.5×10^{-3} M, (B) 5×10^{-3} M, (C) 7.5×10^{-3} M,

(D) 10^{-2} M, (E) 1.25×10^{-2} M, (F) 1.5×10^{-2} M,

(G) 1.75×10^{-2} M, (H) 2×10^{-2} M, (I) 2.25×10^{-2} M,,

(J) 2.5×10^{-2} M, (K) 2.75×10^{-2} M, (L) 3×10^{-2} M.


A
530
D
408
B
527
E
314
C
512
F
196
% Cell population
Cell Length (x100 µm)

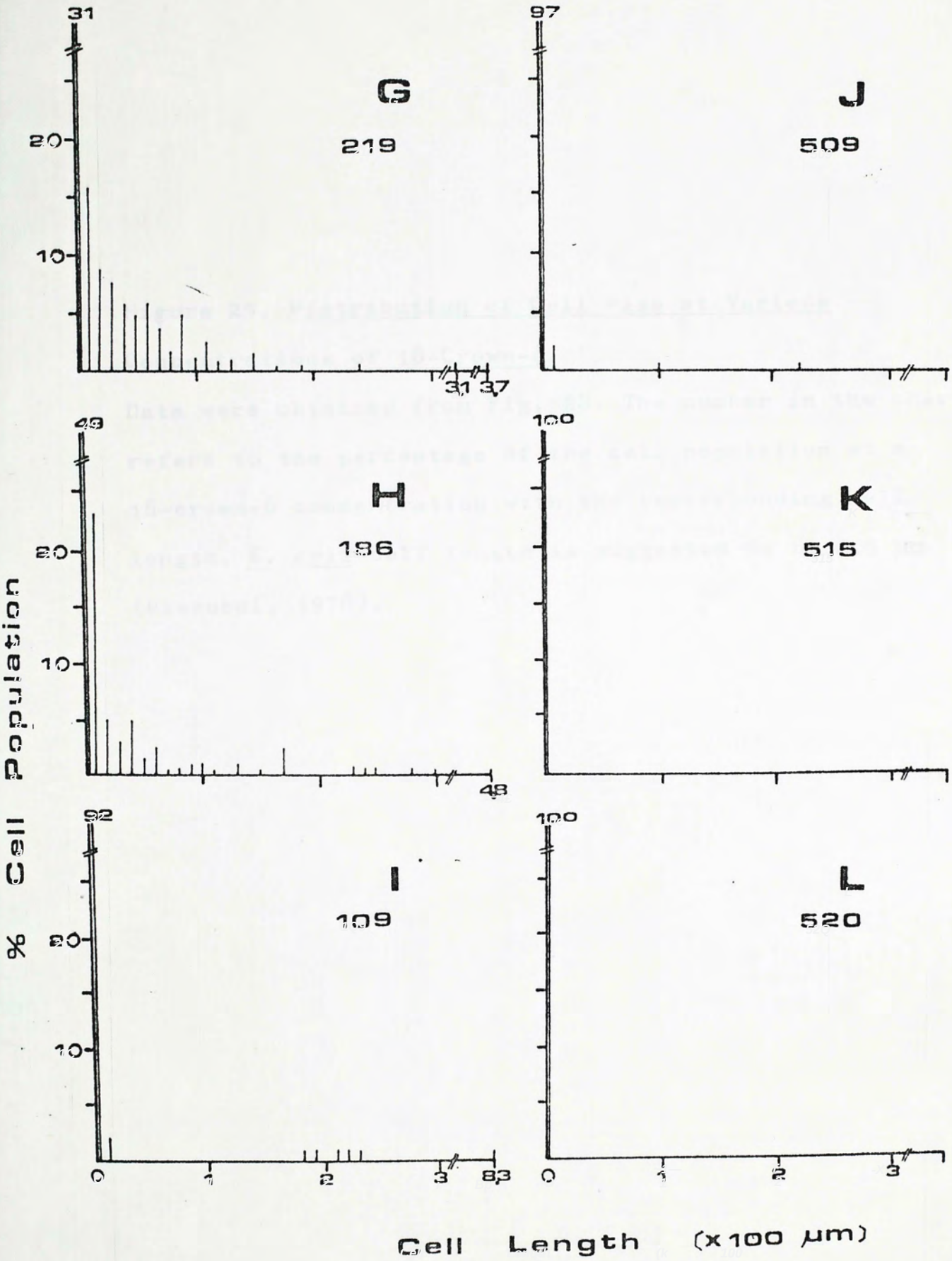
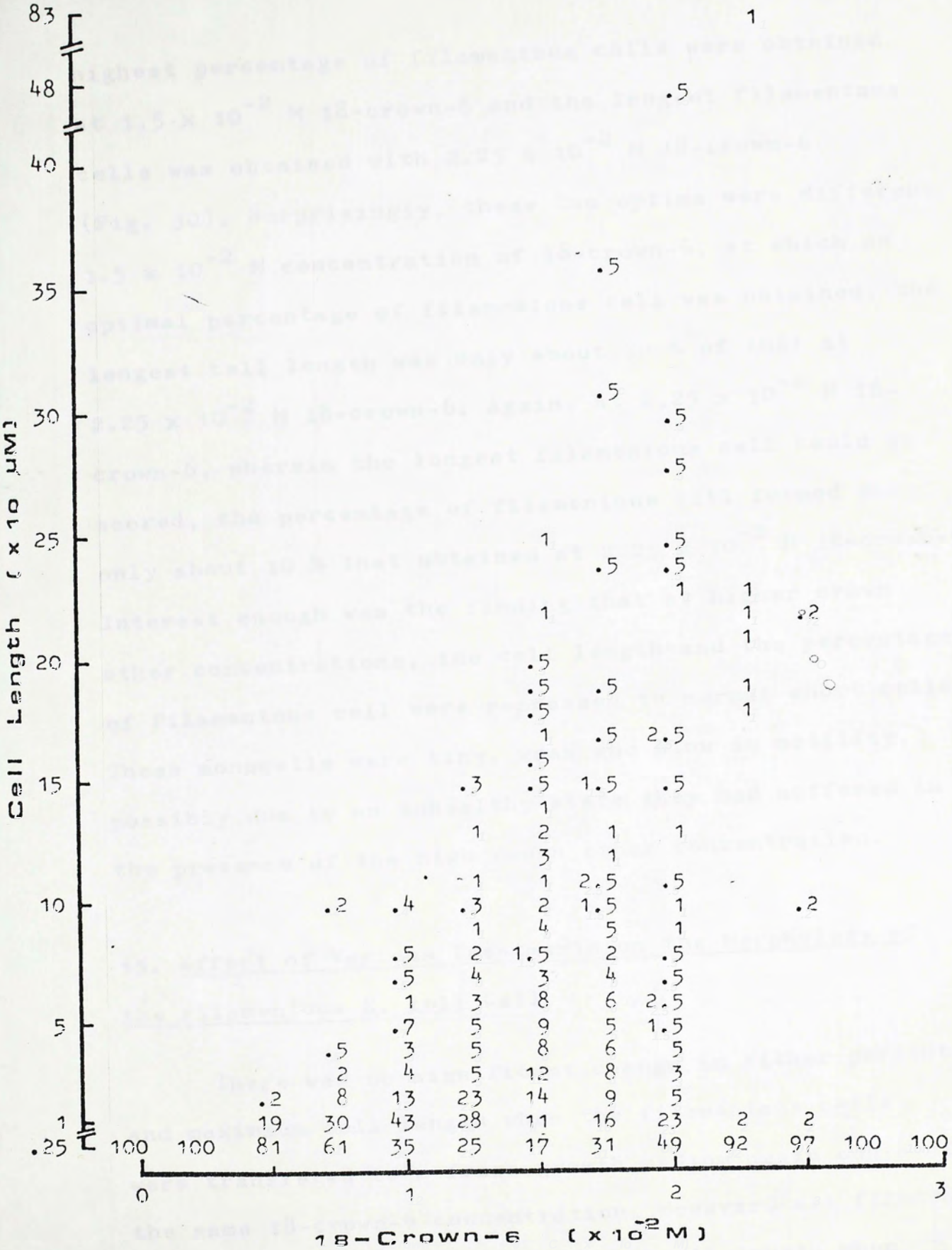

G
219
J
509
H
186
K
515
I
109
L
520
31
97
49
100
92
100
20
10
0
1
2
3
31
37
48
83
% Cell Population
Cell Length (x100 µm)

Figure 29. Distribution of Cell Size at Various Concentrations of 18-Crown-6.

Data were obtained from Fig. 28. The number in the chart refers to the percentage of the cell population at a 18-crown-6 concentration with the corresponding cell length. *E. coli* cell length is suggested to be 2.5 μm (Pierucci, 1978).


Cell Length (x 10 μM)
83
48
40
35
30
25
20
15
10
5
1
.25
18-Crown-6 (x 10⁻² M)
0
1
2
3

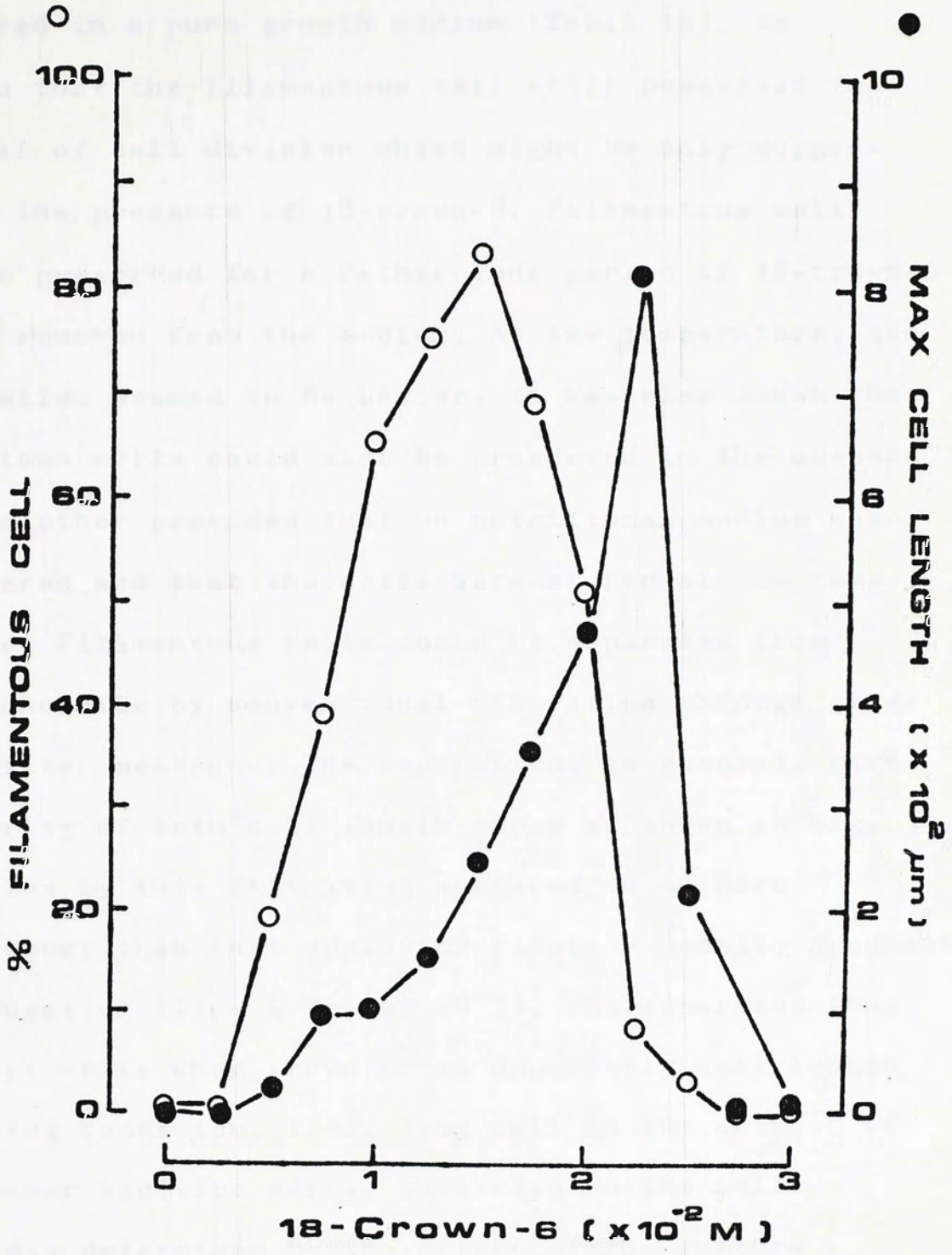
highest percentage of filamentous cells were obtained at 1.5×10^{-2} M 18-crown-6 and the longest filamentous cells was obtained with 2.25×10^{-2} M 18-crown-6 (Fig. 30). Surprisingly, these two optima were different. 1.5×10^{-2} M concentration of 18-crown-6, at which an optimal percentage of filamentous cell was obtained, the longest cell length was only about 30 % of that at 2.25×10^{-2} M 18-crown-6. Again, at 2.25×10^{-2} M 18-crown-6, wherein the longest filamentous cell could be scored, the percentage of filamentous cell formed was only about 10 % that obtained at 2.25×10^{-2} M 18-crown-6. Interest enough was the finding that at higher crown ether concentrations, the cell length and the percentage of filamentous cell were repressed to normal short cells. These monocells were tiny, weak and slow in motility, possibly due to an unhealthy state they had suffered in the presence of the high crown ether concentration.

15. Effect of Various Treatments on the Morphology of the Filamentous *E. coli* Cell

There was no significant change in either percentage and maximaum cell length when the filamentous cells were transfered to a fresh growth medium again containing the same 18-crown-6 concentration. However, all filamentous cells returned to give normal cell length when

Figure 30. Dose Response of 18-Crown-6 on Percentage and Cell Length of Filamentous E. coli Cell.

Data were taken from Fig. 28. Filamentous cells refered to those which were longer than monocells. Maximum cell length refered to the length of the longest filamentous cell registered in the population.


% FILAMENTOUS CELL
MAX CELL LENGTH (x 10² µm)
18-Crown-6 (x10⁻² M)
0
20
40
60
80
100
0
2
4
6
8
10
0
1
2
3

recultured in a pure growth medium (Table 16). It appeared that the filamentous cell still possessed the potential of cell division which might be only suppressed by the presence of 18-crown-6. Filamentous cell could be preserved for a rather long period if 18-crown-6 was not removed from the medium. At low temperature, the preservation seemed to be better. It was also shown that filamentous cells could also be preserved in the absence of crown ether provided that no nutritional medium was encountered and that the cells were stored at low temperature. Filamentous cells could be separated from short monocells by conventional filtration through glass fiber filter membrane. The separation, in general, gave good purity of both cell length types as shown in Fig. 31. Separation by this filtration appeared to be more satisfactory than that employing Ficoll - density gradient centrifugation (Iida & Koibe, 1977). The separated long and short cells when grown in an oppositely cell length generating condition, i.e., long cell in the absence of crown ether and vice versa, gave rise to the cell morphology determined by the nature of the culture medium as expected. The short monocell gave rise to a population of long filamentous cell mixed with short monocells, indicating presumably no selection of mutant was achieved by the filtrat on.

Table 16. Alteration of the Cell Morphology of Crown Ether Induced Filamentous Escherichia coli Cells under Various Treatment*

Treatment	Observation
1. Recultured in fresh tryptone broth with identical 18-Crown-6 concentration.	No observable change in size distribution.
2. Recultured in fresh plain tryptone broth.	After approximately 6 generations, all filamentous cells disappeared, probably transformed to normal monocell by cell division.
3. Kept at room temperature for one week.	Slight reduction in size distribution. (decrease in cell length and percentage of filamentous cell).
4. Kept at 0-4°C for one week.	No observable change in size distribution.
5. Suspended the washed cells in distilled water containing no 18-Crown-6 and maintained at 0-4°C for one week.	No observable change in size distribution.
6. Recultured the "long" cells separated from the crown ether induced filamentous population in pure tryptone broth culture[a].	Reappearance of 'short' monocells.
7. Recultured the 'short' cells separated from the crown ether induced filamentous population in tryptone broth supplemented with 10^{-2}M 18-Crown-6.	Reappearance of 'long' filamentous cells.

*The cells were precultured to filamentous in a tryptone broth containing 10^{-2}M 18-Crown-6 (approximately 6 generatins). These cells were transferred to the new conditions, as depicted in treatment. Cell washing to remove crown ether if necessary was done with centrifugation (3 washings).

[a]The separation was done with glass fiber filter membrane as described in Materials and Methods.

Figure 31. Separation of 'Long' Filamentous Cells and 'Short' Monocells from an 18-Crown-6 Induced Filamentous E. coli.

The separation was done as described in Materials and Methods. (A) Monocells in the filtrate, (B) filamentous cells collected from the filter membrane. The objects appeared in the photo were 600 folds amplified.

A

B

16. Effect of Cation Concentration on Filamentous E. coli Cell

The effective concentration range of 18-crown-6 for filamentous cell formation varied with the growth medium (Table 17). No filamentous cell was observed in a defined minimal medium without potassium and sodium. A change in either potassium or sodium ion concentration shifted the effective concentration in producing filamentous cells. In tryptone broth, an addition of potassium chloride, upshifted the effective concentration range while the presence of an extra amount of sodium ion restricted the effective concentration range to the lower portion of that in the control. This observation was also true for minimal medium. As indicated in the growth curve study, the presence of potassium shifted the effect to higher crown ether concentration, no matter the effect was toxicity (in growth) or filamentous cell formation.

A study of this modifying effect in other alkali cation was shown in Table 18. At a chosen 18-crown-6 concentration, addition of sodium led to a gradual reduction in filamentous cell percentage and cell length while the addition of potassium ion, on the other hand, abruptly repressed filamentous cell formation. Other

Table 17. 18-Crown-6 Induced Filamentous Escherichia coli Cell Formation in Various Growth Media*

Growth Medium[a]	Potassium Content (M)	Sodium Content(M)	Filamentous Cell Formation	Effective 18-Crown-6 Concentration (M)
Tryptone broth	7.0×10^{-4}	1.2×10^{-2}	+	5.0×10^{-3} - 2.5×10^{-2}
Tryptone broth	1.0×10^{-1}	1.2×10^{-2}	+	1.9×10^{-2} - 3.7×10^{-2}
Tryptone broth	7.0×10^{-4}	1.1×10^{-1}	+	9.0×10^{-3} - 1.9×10^{-2}
Ammonium phosphate minimal medium	0.0	0.0	-	-
Potassium phosphate minimal medium	1.6×10^{-1}	0.0	+	1.9×10^{-2} - 7.5×10^{-2}
Sodium phosphate minimal medium	0.0	1.6×10^{-1}	+	9.0×10^{-3} - 1.9×10^{-2}

*The concentration of 18-Crown-6 employed ranged from 1.0×10^{-3}M to 0.1M and the test was done in 2-fold serial dilition.

[a]Chemical composition of the growth media were described in Materials and Methods.

Table 18. Effect of Various Cations on the Gross Morphology of Escherichia coli in the Presence of 18-Crown-6*

Cation[a]	Filamentous Cell Formation in Tryptone Broth with Various Cation Concentrations (M)				
	Control‡	0.1	0.2	0.3	0.4
K^+	+++++[b]	-	-	-	-
Na^+	+++++	++++	+++	++	+
Rb^+	+++++	+++	+	-	-
Cs^+	+++++	++	-	cell death	cell death
Li^+	+++++	+++	+	-	-

*A concentration of 10^{-2}M 18-Crown-6 was supplemented in growth media.

[a]Cation chloride salt was added to the original growth medium supplement with 10^{-2}M 18-Crown-6 for cell growth.

‡Control growth medium was 1% tryptone broth which contained 7×10^{-4}M Potassium and 1.2×10^{-2}M Sodium ion

[b]'+' symbol refers to filamentous cell formation. A higher number of this symbol indicates a higher filamentous cell population as well as longer cell length.

ions tested fell in between potassium and sodium.

17. Division of Filamentous E. coli Cell

It looked as if the filamentous cell was formed because of decreased efficiency in cell division, but not because of a complete loss of its dividing property In liquid culture, it was not possible to identify the individual cell thus making a correlation between the parent long cell and its segregated progenies impossible. On agar plate support, at dilute cell population, nevertheless, it was feasible to follow the fate of a filamentous cell population. From the sequential events shown in Fig. 32, the cell division site were found to be randomly distributed along the filamentous cell.

18. Colony Morphology Formed by Filamentous E. coli Cell

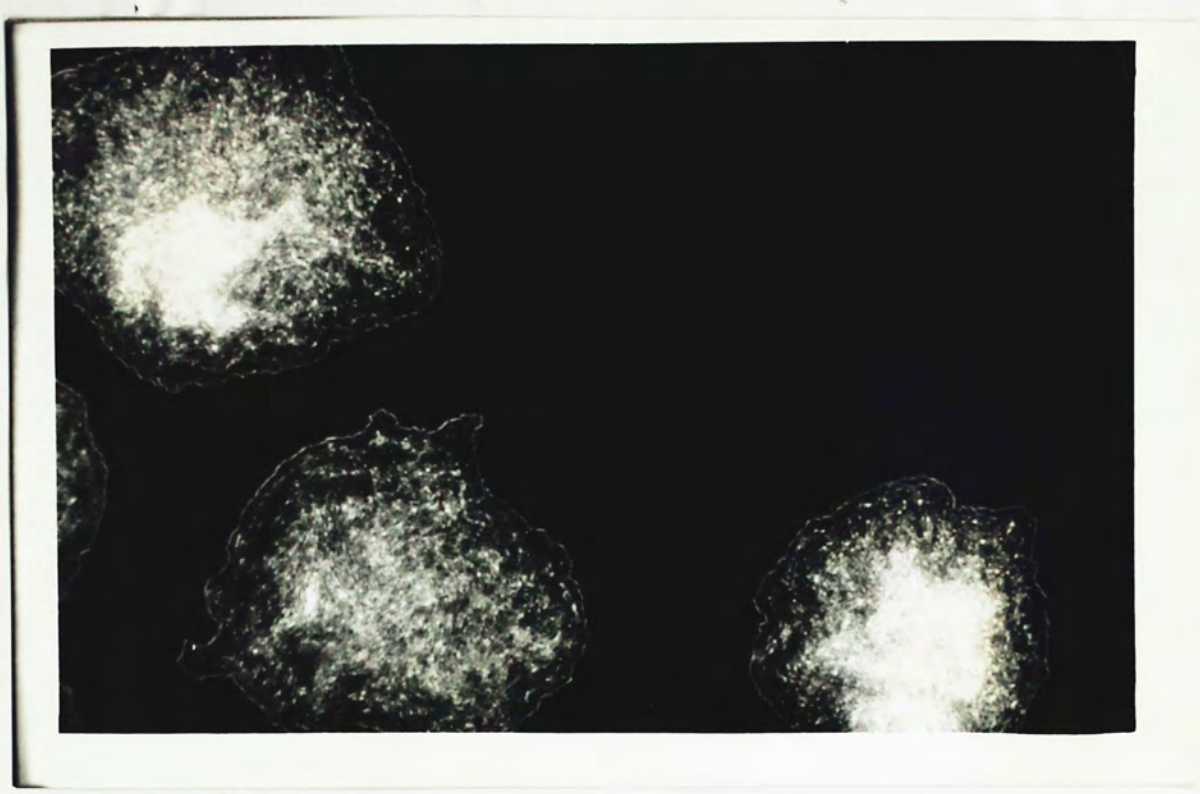
Normally grown E. coli cells formed circular colonies with smooth edge on tryptone agar plates (Fig 33, A). However, the filamentous cell, probably because of the irregular orientation and spontaneous, random cell segregation, produced irregular colony shape, with frimbreate edge and wavy trace on the surface of the colony (Fig. 33, B).

Figure 32. Cell Fission in Filamentous E. coli Cell Grown on Agar Support.

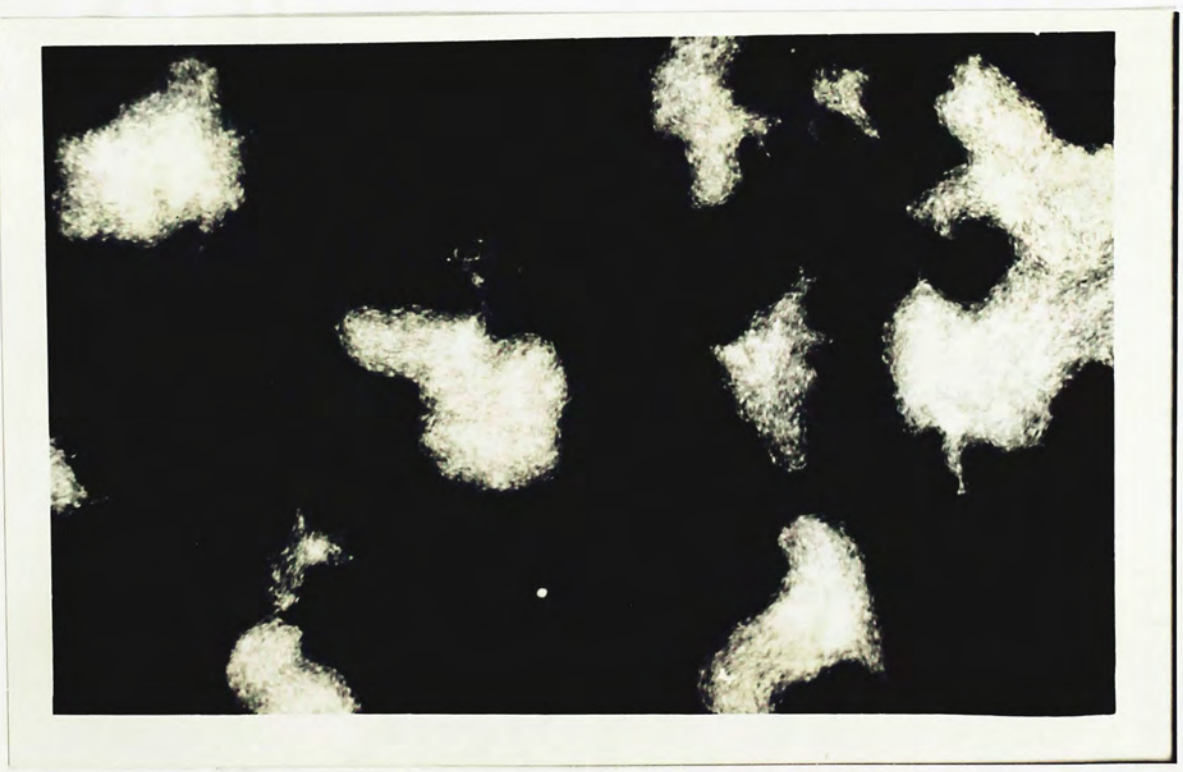
Filamentous E. coli cells prepared by growing in 10^{-2} M 18-crown-6 supplemented tryptone broth were spreaded onto the tryptone agar plate containing the same concentration of 18-crown-6. Photographs were taken at an interval of one hour from a fixed positon above the plate (photo A to D). Dark field illumination was used and the magnification was 200 folds.

Figure 33. Microcolony Morphology Formed by Normal and Filamentous E. coli Cells.

E. coli were streaked on both tryptone plates (1.5 % w/v agar) with or without 10^{-2} M 18-crown-6 supplemented and incubated overnight at 25°C. Microcolonies formed by noraml E. coli cells in tryptone plate (A) and by filamentous cells in 18-crown-6 supplemented plate (B) were photographed at 200 fold of amplifications.



A



B

19. Whole Cell Protein Separation Pattern of Normal and Filamentous *E. coli* in SDS Polyacrylamide Gel Electrophoresis

The formation of filamentous bacterial cells in the presence of 18-crown-6 was an abnormal cell growth phenomenon, probably due to a defect in the cell division system. This cell division defect might have been resulted from a missing of a cellular component(s) or an alteration in a cellular component(s) which was adequately needed for normal cell division. For this reason, the protein patterns of the normal and the filamentous cells were examined.

It appeared that the whole cell protein subunits were quite stable. As shown in Fig. 34, the normal *E. coli* whole cell protein prepared by cell lysis either through sonication or lysozyme digestion gave close to identical band patterns in SDS - PAGE. Since the two cell lysis methods were based on different principles, the result thus indicated that the probability of any protein fragmentation in the preparation of samples must be exceedingly small.

When whole cell protein of normal and filamentous cells were analysed, it was found that besides the

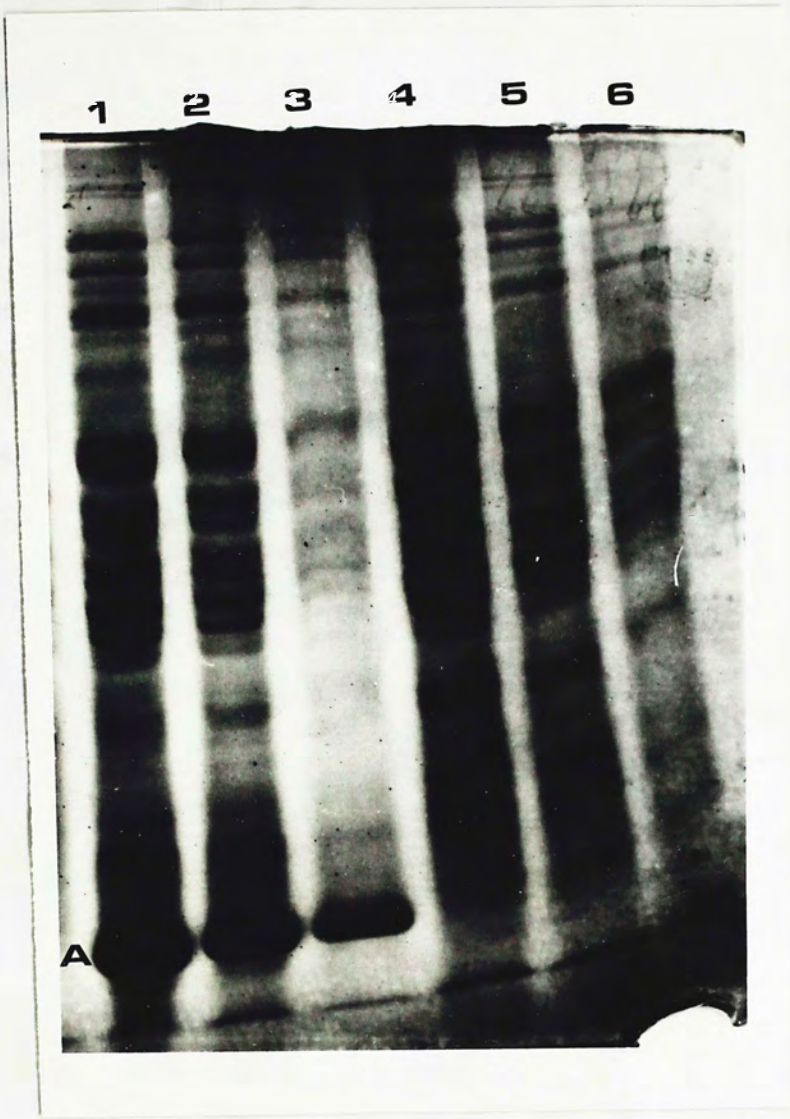


Figure 34. Whole Cell Protein Pattern of Tryptone Broth Grown E. coli

Bacterial whole cell protein was prepared by cell lysis either through lysozyme treament (slot 1 to 3) or through sonication (slot 4 to 6). A 12.5 % SDS polyacrylamide slab gel was used. Protein band A is lysozyme.

similarity of the protein pattern, three distinct protein bands were absent in filamentous cells (Fig. 35). The molecular weight of these three protein bands A, B, and C were determined by comparing the relative motilities with the commercially available protein markers for PAGE studies and found to be 120,000; 70,000 and 42,500 correspondingly (Fig. 36).

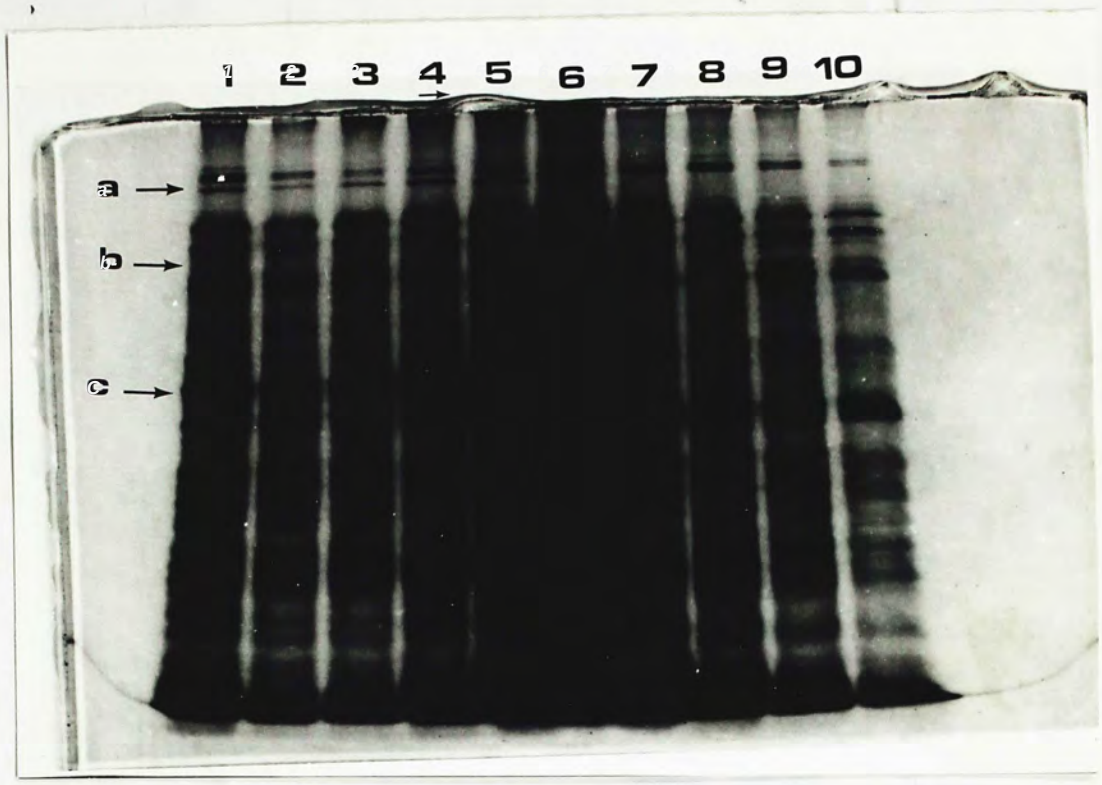


Figure 35. A comparison of the Whole Cell Protein Pattern of Normal and Filamentous E. coli Cells.

Whole cell protein of tryptone broth cultured E. coli in the absence (slot 1 to 6) and presence of 1.5×10^{-2} M 18-crown-6 (slot 7 to 10) were separated by SDS polyacrylamide gel electrophoresis. Filamentous cells were formed in 18-crown-6 supplemented medium. The bacterial cell protein were prepared by sonication of cells as described in Materials and Methods. A 10 % SDS polyacrylamide slab gel was used. Note the disappearance of bands a, b and c in the filamentous cell protein pattern.

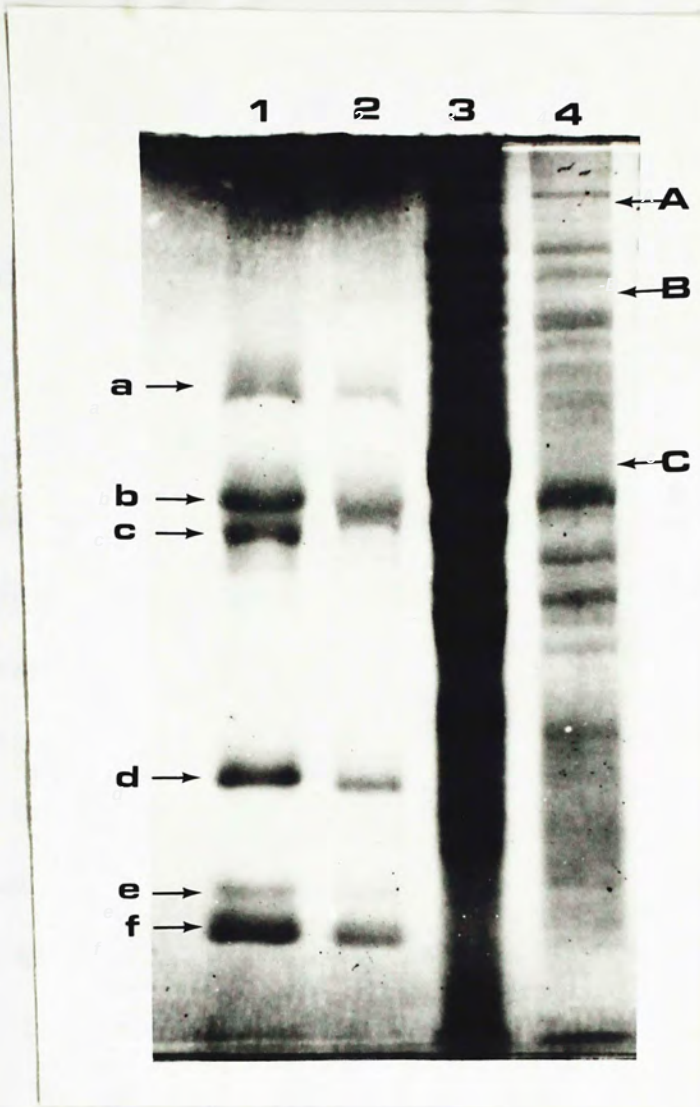


Figure 36. Molecular Weight Determination of the Protein Band **A** , **B** and **C** Absent in the Filamentous *E. coli* Cell. Standard protein markers (slot 1 and 2) were included in SDS - PAGE studies with the whole cell protein of normal (slot 3) and filamentous (slot 4) *E. coli* cells. The protien markers were (**a**) bovine albumin, (**b**) pepsin, (**c**) ovalbumin, (**d**) trypsinogen, (**e**) β-lactoglobulin, and (**f**) lysozyme. Protein bands **A** , **B** and **C** are the three missing protein subunits in filamentous cells.

IV. DISCUSSIONS

1. Complexability of Crwon Ether towards Alkali Metal Ions

The complexability of crown ether towards different kinds of alkali metal ions is determined by many factors, including the relative size of the cation and the cavity of the crown ether; the number, the arrangement, and the basicity of oxygen atoms in the crown ether ring, the steric hindrance, the tendency of the cation to associate with the solvent and others. These factors taken an a whole, affect the stability of the complex (Pedersen, 1967; Christensen et al., 1971; Pedersen & Frensdorff, 1972; and Ng, 1979).

For 1:1 crown ether metal ion complexes , as all those appeared in this study, the metal ion is located at the centre of the crown ether cavity which makes the relative sizes of the cation and the cavity the key factor in determining the stability of the complex. Metal ions of sizes either too small or too big will not form stable complexes. The extraction equilibrium constants presented in the results show the trend of this stability. The members of 18-crown-6 family with a cavity diameter around 2.6 - 3.2 Å, show the highest

complexability towards potassium ion (diameter 2.66 Å), and 15-crown-5, with cavity diameter of 1.7 - 2.2 Å, forms the most stable comple with sodium ion (diameter 1.9 Å).

The members of the 18-crown-6 family do show minor variations in their extraction equilibrium constants. This variation may have stemmed from the substitution on the skeleton, thus leading to a change in the electron density available at the complexing oxygen. Crown ether with substituted groups containing only aliphatic carbon atoms has higher basicity than that having substituted group with aromatic carbon atoms. This is illustrated by the high extraction equilibrium constant of dicyclohexyl-18-crown-6 towards metal ions, while much smaller value for benzo-18-crown-6. The presence of an electrom donating methyl group on the benzene ring of the crown ether, however, increases the basicity of the oxygen atoms which has been weaken by the arometic ring. This is also reflected on the complexation property as observed in the values K_e determined for 4'-methyl-benzo-18-crown-6 and benzo-18-crown-6.

Complexation may involve a spatial change of the crown ether ring to achieve maximal interaction between the cation and the binding oxygen atoms. Any steric

hindrance introduced in the crown ether ring which is unfavorable for a conformational change will decrease the stability of the complex (Lehn, 1973). A lower value in benzo-18-crown-6 than the parent 18-crown-6 may be partly resulted from a steric hindrance introduced by the presence of bulky benzene ring.

For the monovalent alkali metal ions, the smaller the ionic size, the higher will be its solvation. In general, the solvation energy is an inverse function of the ionic diameter in a given group of elements. For highly solvated cations, more energy is needed for the crown ether to compete with the solvent in complexation. The much lower crown ether complexability to lithium ion clearly illustrated this factor.

A rule of thumb for these complexes is that stability of the complex usually reflects the complexability. Nethertheless, ion selectivity is a different story. In general, the factors that affect the stabilities of crown ether-cation complexes are also factors that influence the selectivity of the corresponding crown ether towards the ion. A rigid crown ether ring which suits only one specific cation and discriminates against other cations will be highly selective. The fact that similar selectivity trend exists in all 18 crown-6 members indicates

that cavity versus ion size effect is the dominant factor in determining complexability. Minor difference among 18-crown-6 family may thus be resulted from the steric factor or the reduction of oxygen basicity in the ring, as described in earlier paragraphs.

In biological systems, the distribution of sodium ion and potassium ion is of great physiological significance (Harold & Altendort, 1974). In order to examine the physiological effect of crown ether on biological systems, an understanding of the interactions of these two ions with crown ether is required. This includes a study of the nature of rubidium in respect to potassium ion. This study indicates that for chemical interactions, all members of the 18-crown-6 family have a high preference on complexing with potassium. Of great interest is the finding that the parent 18-crown-6 has the highest selectivity for potassium among the family. This as well as its commercial available has placed it in a position as the main crown ether used in studying its effect on microbial physiology. And for a system involving the 18-crown-6 family, it is also shown that rubidium can be used to mimic the effect of potassium without much alteration. This provides a sound rational for later studies with rubidium in defining potassium distribution.

It must be pointed out here that for crown ether facilitated ion transport through organic layer, besides the effect of the stability of the complex, the solubility of the crown ether in the binary phase also affects the extraction efficiency (Pedersen & Frensdorff, 1972). Furthermore, the extraction method with water-chloroform binary system should be considered as a primitive artificial membrane model for crown ether facilitated ion transport only. Biomembranes must be much more complicated. Early studies by Eisenman et al.; Tosteson, and Lardy, however, show that 18-crown-6 compounds do positively facilitated the potassium permeability in both the artificial as well as the natural membranes (Eisenman et al., 1968; Tosteson, 1968; Lardy, 1968). This data will be taken as some fundamental properties of the crown ether in explaining the phenomenon observed in later studies. As the chemical composition of bacterial membrane has proved to be in general similar to that of other biological membranes (Martin & MacLeod, 1971), it seems that the facilitated ion permeability by crown ether will not be greatly violated from that studied on other natural membranes.

2. Toxicity of Crown Ether on E. coli

The study on MIC susceptibility test showed that a similar toxicity in E. coli was found among the 18-crown-6 family and a less toxicity for small ring compound 15-crown-5. As potassium is the most essential one in biological systems among the alkali metal ions (Lester, 1958), this might reflect the efficiency of these crown ethers in transporting potassium and hence the effect of these crown ethers in interfering potassium ion distribution in the bacterial cells. However, it should also be noted that MIC test merely suggests a preliminary indication on chemical toxicity. The fact that the MIC results are consistant with the growth curve study demonstrates the general application of convenient MIC test in screening toxic chemicals.

3. Toxicity of 18-Crown-6 to Microorganisms

It has been suggested that ionophores are often mildly toxic to microbial organisms while extremely toxic or have strong physiological effect on higher organisms (Pressman, 1976). For crown ether, similar result was obtained. This further implicates that the toxicity of crown ether might be closely related to their ionophoretic property.

Among the organisms studied, eukaryotic protozoa Tetrahymena thermophila is the most susceptible species in 18-crown-6 toxicity, while all other common bacterial species studied are about ten folds less. It is of great interest to find Halobacterium cutiritium, a controversial prokaryotic species with more close kinship to lower eukaryotes demonstrates a crown ether susceptibility behavior close to the protozoa. In literature, the MIC value of dicyclohexyl-18-crown-6 on algae, Chlamydomonas reinhardi is about 2.7×10^{-4} M when grown protoautotrophically or mixotrophically and is even lower, at 5.4×10^{-5} M on heterotrophical cells (Mottley & Griffiths, 1977). Although this result is obtained by colony formation on agar plates, the MIC value thus determined is expected to be close to that obtained from liquid culture MIC value. In fact, a comparison of the MIC vale values studied by both methods with bacteria showed that the MIC value indicated by colony formation on agar plate was about 2 folds higher than that by turbidity in liquid culture. It is obvious that crown ether is more toxic on algae than on bacteria. Furthermore, in rat, dicyclohexyl-18-crown-6 exhibits as approximate lethal dose (ALD) of 300 mg/Kg by ingestion, and exhibits an even more higher toxicity by absorption through skin, causing fatality at a dosage of 130 mg/Kg (Pedersen, 1967

& 1972). And in beagle dogs, 18-crown-6 at a dosage of 500 mg/Kg administered orally induced symtoms such as tremulous movement, salivation and paralysis of the hind legs (Takayama et al., 1977). All these toxicity values when expressed in MIC equivalent would provide a supporting evidence that higher organisms are more vulnerable to crown ether toxicity.

Since the crown ether effect is likely to be membrane associated, the differential susceptibility of the organisms might be related to their membrane structure or membrane chemistry. As regards membrane structures and chemistry, they are somewhat different between prokaryotes and eukaryotes (Wallach & Gordon, 1968). This may probably help in explaining a toxicity difference between the two cell types which are found in the study.

4. Effect of Crown Ether on E. coli Growth

It appears that besides the physical disruption of the cell integrity at very high concentrations, no obvious destruction can be seen to be exerted by the ionophoretic crown ethers up to 10^{-2} M concentration on non-growing bacterium E. coli. For growing cells, an inhibiting effect, however, can be witnessed in all three phases in the growth curve. Chemicals which affect

the growth curve have been extensively reported. They vary from ion chelators (Collins et al., 1979), microbicides (Yamada et al., 1977), surface active agents (Lamikanra & Allwood, 1976) and ionophores (Harold & Ba Baarda, 1967 & 1968). The crown ether toxic effect, as it delays the starting of exponential growth and decrease the growth yield, is in general similar to most of the adverse effects rendered by the chemicals mentioned above. In spite of some minor variations in the effect of individual crown ether, it seems that a common inhibition mechanism is underlying the change in bacterial growth pattern.

It is found that a fraction of the cells was killed by the crown ether (the viability test), that the added crown ether was not significantly degrated after considerable cell growth. (solvent extraction studies), and that the exhausted growth medium contained no potent toxicant. The survived cells in the population must somehow achieve some means by which the crown ether toxicity had been overcome. This rescuing effect is presumably an energy dependent process as shown by the reduction in the final growth yield reflecting an early development of nutrient exhaustion. This rapid nutrient exhaustion is due possibly to an excess energy usage by

the survivors. When cells are allowed to be subcultured in a non-stressed medium without crown ether, the energy depending process is no longer in demand and thus on reculturing in a crown ether supplemented medium, a growth lag reappears. Moreover, if the survivors at their logarithmic phase, are immediately transfered to a non-stressed medium, a detectable shortening in their doubling time is demonstrated.

In bacterial systems, other potassium specific ionophores, such as valinomycin, gramicidin, nigericin and monactin, have been reported to have similar inhibition in bact rial growth. This inhibition arises from a loss of potassium ion in the cells, and have been demonstrated to be reversed by the addition of excess potassium ion (Harold & Baarda, 1967 & 1968). Similar potassium rescuing effect was demonstrated on the growth inhibition by 18-crown-6, as indicated by a shortening of the crown ether induced lag and the reverse of the stationary population reduction. However, it is worthwhile to point out that this rescue is only effective with the parent 18-crown-6 and not its derivatives. Bacteria generally accumulate high concentration of potassium in the cell from its extracellular environment by a specific active transport system involving

the consumption of energy (Harold & Altendorff, 1974). This potassium active transport is suggested to be more efficient in E. coli (Lester, 1958). As a result, a high potassium concentration gradient is established between the intracellular fluid and the outside environment due to the impermeability of lipophilic plasma membrane (Schultz et al., 1962; Ginzburg et al., 1970). The effect of 18-crown-6 can then be interpreted to mean an increase of potassium permeability through the plasma membrane by crown ether, thus upsets the potassium barrier. Hence, an addition of potassium to the medium at an unusual high concentration will readjust the thermodynamic equilibrium and bring the cells back to a state where the intracellular potassium ion concentration is comparable to normal intact cells. The presence of sodium ion does not seem to aid in correcting the upset gradient. The result in solvent extraction study indicates that 18-crown-6 has a higher selectivity towards potassium over sodium. This may be part of the explanation to the uniqueness of the parent 18-crown-6 in this potassium rescue action. The fact that this simple reason cannot offer good explanation to the effect of other 18-crown-6 derivatives indicates the possible complicating factors involved in this phenomenon.

So far as the reduction of cellular potassium is concerned, it has been reported that when potassium in the growth medium was lowered to levels insufficient for the maintenance of optimal bacterial growth, activity of the membrane ATPase increases in accompany with an elevated potassium uptake as well as rate of cellular glycolysis (Abrums & Smith, 1971). Apparently, increase of ATPase activity is an adaptive response of the bacterial cells to a decreased intracellular potassium concentration. The enzyme ATPase has been suggested to be related to potassium translocation by ATP hydrolysis (Harold et al., 1969a & b). Hence, if the expression of the crown ether toxicity is through enhanced potassium leakage through cell membrane, as more clearly indicated by the parent 18-crown-6, then it is reasonable that the possible energy dependent adaptive process of the bacterial cells as discussed in earlier paragraphs, might involve the enzyme ATPase. This is further supported by the study that in rat liver mitochondria, 18-crown-6 compound induce ATPase activity by enhanced ATP hydrolysis, with simultaneously a facilitated potassium permeability through the mitochondrial membrane (Lardy, 1968).

5. Effect of 18-Crown-6 on ^{86}Rb Transport through *E. coli* Cell Membrane

E. coli possesses an active transport system with a high affinity for potassium (Epstein & Schultz, 1965 & 1966) and is able to maintain very high concentration gradient of intracellular potassium (Weiden, 1967). This transport process is strictly energy dependent (Zarlengo & Schultz, 1966) and apparently no potassium transport through the cell membrane is found unless supplied with metabolic energy such as glucose (Harold & Baarda, 1968). 18-Crown-6, at a concentration of 10^{-2} M (the highest concentration generally used in the study on bacterial physiological change), reduced only slightly the ^{86}Rb uptake by bacterial active transport system and appeared to have no effect on bacterial cells in the absence of exogenous energy supply. It seems that 18-crown-6 at 10^{-2} M does not affect severely the active transport machinary or is not well demonstrated in the condition studied.

However, according to the leakage study, an enhanced Rb permeability in the bacterial membrane was generated by 18-crown-6 and the effect was much more severe in longer incubation period. This loss of bacterial endogenous ^{86}Rb is probably due either by leakage,

through cell membrane, or by exchange with other cations in the outer medium.

It is consistent with the finding in this study that the presence of crown ether at a concentration which is capable of affecting the ion leakage in ^{86}Rb experiments, also creates all observed growth abnormality such as lag, decrease of logarithmic growth rate and growth yield reduction. It is also reasonable that the presence of external potassium in the medium can change the intracellular potassium concentration thus rescue the bacterial cells——— converting it back to normal growth.

6. Effect of Crown Ether on Bacterial Respiration

Respiration rate is a convenient measurement of the state of the organism since the electron transport chain is very sensitive to xenobiotics. The presence of a toxicant to an organism is usually detected by an adverse change in the organism's respirating pattern long before the organism is being killed.

The fact that a mild respiration inhibition at low crown ether addition appears to suggest that the lipophilic property of the crown ethers might render them to be membrane active compounds and have generated

an interaction with the membrane bound respiratory chain at very low concentration (below 10^{-4} M). However, the other main features of the organism may retain unaltered. But at high crown ether concentration, vital damages are produced so that a drastic decrease in respiration is observed. This effective concentration range and the individual crown ether potency are consistent with that determined by the viability and growth rate tests. The bi-phasic nature of the toxic effect either suggests a composite inhibition taken place or the appearance of another major harmful mechanism to have been turned on at high crown ether concentrations.

7. Crown Ether Specificity in Filamentous E. coli Cell Formation

Among all crown ethers studied, only 15-crown-5 and 18-crown-6 have an effect in inducing filamentous cell formation. This specificity may have stemmed from a loss of ability when the parent crown ether skeleton is substituted or a shift to higher toxicity in the crown ether derivatives such that the organism may be killed before reaching an effective concentration for filamentous cell formation. It should be noted that the effective concentration of bacterial filamentation for

both crown ethers are high and that the toxicity of parent 15-crown-5 and 18-crown-6 is comparatively lower than those substituted ones. These might be an indirect support to the latter proposal for the failure of filamentous cell formation in the presence of substituted crown ethers. However, the data obtained here can by no means rule out the possibility of the former proposal.

3. Conditions Affecting the Bacterial Filamentous Morphology

Study of 18-crown-6 on various bacterial species as well as other findings of filamentous bacteria, indicate that only rod-shape bacteria possess ability to form filamentous cell. The shape of bacteria, either coccus or rod-shape, appears to be correlated to a/ the chemical components in the cell wall (Petros & Lynch, 1975), b/ the type of septum formation and c/ the direction of cell separation(Amako & Umeda, 1979). Hence, it seems reasonable that filamentation was observed only in rod shaped bacteria as demonstrated in this study. In rod-shaped bacterium, cell elongation is either unipolar or bipolar (William, 1959; Adler & Hardigree, 1964). The elongating rod-shaped cell has new cell wall materials added onto its division site (Cole 1965; Cole & Halm, 1962). This

process involves a degradation of the old capsule by lytic enzymes (Shockman *et al.*, 1967) as well as an addition of newly synthesized materials on the capsular network by synthetic enzymes (Osborn, 1969).

It is possible that inhibition on either the degrading or the synthesizing enzymes might lead to a failure in cell elongation. On the other hand, cell division is initiated by a complete replication of nuclear DNA (Clarke, 1968; Cooper & Helmstetter, 1968) which is followed by septum formation before the release of the new progenies (Rogers, 1970). The filamentous *E. coli* cell, being polynucleated as demonstrated by histochemical study, indicates that the cell can elongate and its DNA synthesis is not inhibited. And it has been reported that filamentous cells induced by the inhibition of DNA synthesis would result in the exitance of anucleated filaments (Jeener & Jeener, 1952; Zamenhof *et al.*, 1956). However, in the crown ether induced filamentous cells, nuclear structures are regularly distributed along the body while cellular septa are lacking lacking. It seems that the filamentous cell with *E. coli* as a typical example is formed *not* because of the lack of genetic material but because of insufficient septa synthesized. The internulear spaces may represent the

locations where septa would be formed if such formation were permitted.

The filamentous E. coli cells still possess a lower level of cell division property. It appears more appropriate to describe the filamentous cell as a cell state in which the cell elongation is still working but the cell septation is handicapped by the presence of 18-crown-6. At each 18-crown-6 concentration, the cell length distribution, percentage of filamentous cell and cell length may reflect a resultant of the 18-crown-6 on both cellular growth processes. As E. coli suspended at a higher than effective concentration produces only monocells, it is possoble to give an explanation based on a differential concentration effect of 18-crown-6 on the two cellular growth processes. At lower affective concentrations, cell elongation is not significantly affected while cell septation is affected, resulting in the formation of filamentous cells. At higher effective concentrations, however, the inhibition on cell elongation begins to take effect. It gives rise to tiny monocell with sick features. Further evidences on this differentiate effect is supported by the study with penicillin. At low concentrations penicillin, only cell division is inhibited results in elongated cells, whereas at high concentrations,

inhibition of both cell division and elongation occurs, and followed by cell lysis (Schwarz et al., 1969)

Both the production of filamentous cell in E. coli and the resumption of monocell from a filamentous population require a growth process. In a low growth condition (low temperature) or a starved condition (suspended in pure water), filamentous morphology remains. Variation of alkali metal ion in the growth medium changes the E. coli effective concentration in inducing filamentous cell formation. The effect is most powerful with potassium and least with sodium ion. This observation indicates further the close relation of the ionophoretic property of crown ether and their physiological effects on bacteria, in both filamentous cell formation and growth curve pattern variations.

All the observations mentioned above suggest that the formation of the filamentous cell probable results from the effect done by some defective component(s) or by the absence of some component(s) which are biosynthesized in the two cellular growth processes. The presence of the alkali metal ion may be serving as some effector in altering the biosynthesis of this component(s), either directly or indirectly. This model gains support by the finding of three mis g protei ids in com-

paring the whole cell protein of normal E. coli cells and filamentous cells.

It is worthwhile to point out that the 18-crown-6 concentrations at which filamentous cells form, alteration in growth curve takes place, radioactive endogenous rubidium leaks, absence of certain major whole cell protein bands, and a drop in respiratory rate are existing in overlapping range. This may either be due to a diversified adverse effect or a single component commonly related to all or most of the above processes is altered by 18-crown-6. There is at present no knowledge as yet to how crown ether leads to such a defect even though so some hints may have been observed that the cation flux involve in the phenomenon.

9. Cellular Feature in Filamentous Cell

The 18-crown-6 induced filamentous E. coli cell has a smooth cell surface. It has few cellular septa On solid support, the filamentous cell devides occasionally to shorter fragments. A unique character of the filamentous cells is their extraordinary cell length. This kind of morphological change is remarkably different from either those irregular cell shape due to capsule damage by xenobiotics, such as ampicillin (Staugaard

et al., 1976), or those E. coli mutants that have lost the ability to exist in normal cell shape (Long et al., 1978). The filamentous cells are motile, and can carry out all normal physiological functions except that needed for cell division.

10. Microcolony of Filamentous E. coli Cells on Agar Support

Growth of E. coli on agar in the presence of 18-crown-6 shows a unique geometry of the microcolony profile. The solid support, differed from the liquid medium, since it takes time for its near environment to exchange chemical substances with the distant environment. This gives rise to problems such as the exhaustion of nutrient and the accumulation of toxic metabolites (Cooper et al., 1968; Jones & Gray, 1978a & b; Palumbo et al., 1971). Generally speaking, each bacterial species forms a unique colony type on agar plate. The colonies differ in size, shape, edge, elevation, internal structure, color and in other characteristics. These characters may be used for the identification and classification of bacteria (Finkelstein & Punyashthite, 1967). E. coli on agar plate forms close to circular microcolonies with fine, granular internal structure and smooth

edge, a typical characteristic colony morphology of the rod shaped bacteria (Drucker & Whittaker, 1971). Micro-colonies formed by filamentous E. coli cell induced by 18-crown-6, however, show wavy interlaced structure with frimbriate edge. Apparently, this feature results from the oriented arrangement of the filamentous cells.

11. Absence of Three Major Protein Bands of Filamentous E. coli in SDS Polyacrylamide Gel Electrophoresis

The cell division defect seems to have stemmed from the lacking of some functional proteins in the filamentous cell division machinary. A comparison of the whole cell protein electrophoretic pattern indicates that the major difference is the absence of some proteins and not the change of protein mobility due to protein alteration since no major new bands appear in the filamentous cell protein pattern. Although three bands A, B and C were obviously shown to be lacking in the filamentous cell, there is no knowledge as to which one or all of these are responsible for normal cell division. Furthermore, some undetected minor protein(s) may also be reponsible. The main feature of the observation here may be a demonstration of the difference of the whole cell protein profile and a clue for further investigations.

V. CONCLUSIONS

Major Findings in This Thesis :

1. Chemical study with the solvent extraction method has shown that crown ethers show different complexability with group IA alkali metal ions correlating to the variations in crown ether ring size and the substituted groups.

2. The toxicity of crown ether is comparatively milder on bacteria than other higher level organisms such as protozoa.

3. All 18-crown-6 compounds exhibit similar toxicity pattern : an induced lag, occasionally slower logarithmic growth rate and a stationary growth yield reduction.

4. The cause of this 18-crown-6 induced bacterial growth lag and growth yield reduction is shown to be caused by a lower viability of the inoculum and a rapid exhaustion of nutrient in the presence of crown ether.

5. The 18-crown-6 effect on bacterial growth is highly crown ether specific : Addition of potassium or rubidium reduces the adverse effect due to the presence of 18-crown-6 but reinforces that due to the substituted 18-crown-6 compounds. On the other hand,

addition of sodium does not generate the same effect.

6. The effect of 18-crown-6 appears to have resulted from an increase in bacterial membrane potassium permeation as shown by labelled rubidium transport studies.

7. In addition, the 18-crown-6 compounds exhibit a bi-phasic inhibition effect on bacterial respiration : slightly reduced respiration at low concentrations and an abrupt drop of respiration at high concentrations.

8. Of all the crown ethers, only the unsubstituted parent 15-crown-5 and 18-crown-6 induce filamentous cell formation in E. coli systems. This effect is only found in rod shape bacteria.

9. Optimal 18-crown-6 concentrations for the production of the highest filamentous E. coli cell population and the longest individual cell are found to be separated.

10. Morphologically, the 18-crown-6 induced filamentous E. coli cell has a body length of approximately a hundred fold or more longer than the normal cell, and is polynucleated and lack of septum.

11. A satisfactory method is described to separate the filamentous bacterial cells from the monocells. This method utilizes glass fibr[illegible] brane and

method utilizes glass fibre filter membrane and is convenient, simple and effective.

12. The filamentous cells can be reversed to normal cell length as shown by growth in the absence of 18-crown-6 however, this filamentous state can be preserved if needed simply by stopping the cell growth.

13. The filamentous cells formation is alkali metal ion dependent : potassium being able to lower the effect of crown ether (i.e. needs a higher effective concentration) and sodium to increase the effect.

14. The filamentous E. coli cell shows abnormal colony morphology : with frimbriate edge and wavy internal structure.

15. Biochemically, the filamentous E. coli whole cell protein pattern shows an absence of three major protein bands in SDS polyacrylamide gel electrophoresis.

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APPENDIX I

Absorption Spectra of Crown Ether - Picrate Salt Complexes Extracted in Chloroform Layer by the Solvent Extraction Method

Picrate salts used : (Fig. 1) KPi, (Fig.2) NaPi, (Fig.3) RbPi, (Fig.4) CsPi, (Fig.5) LiPi.

Crown ethers used : (A) 15-crown-5, (B) 18-crown-6, (C) benzo-18-crown-6, (D) 4'-methyl-benzo-18-crown-8, (E) dicyclohexyl-18-crown-6, (F) without crown ether.

The value at the symbol of the corresponding crown ether refered the dilution fold of the original organic solution for spectrometric measurement.

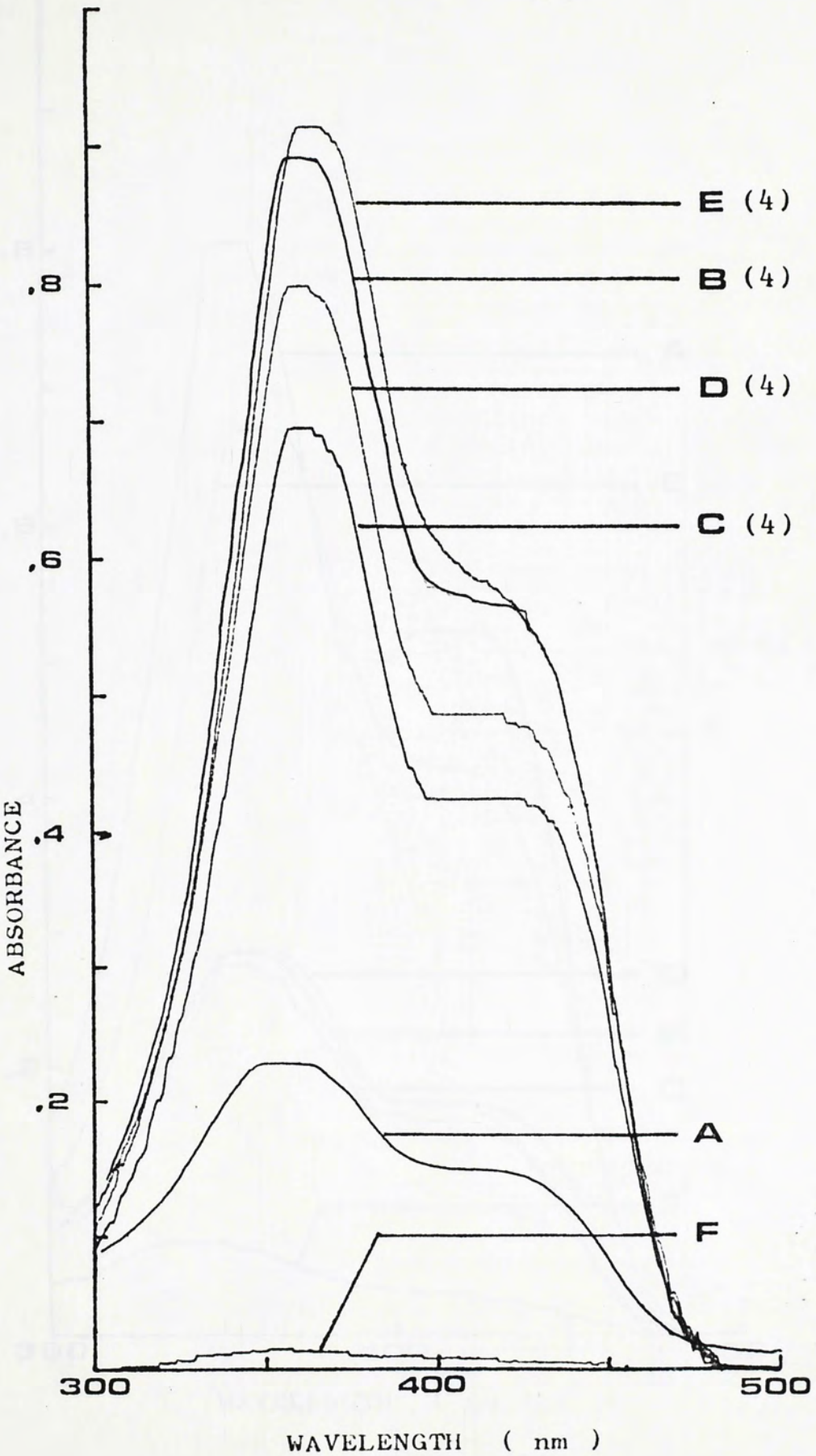


Figure 1.

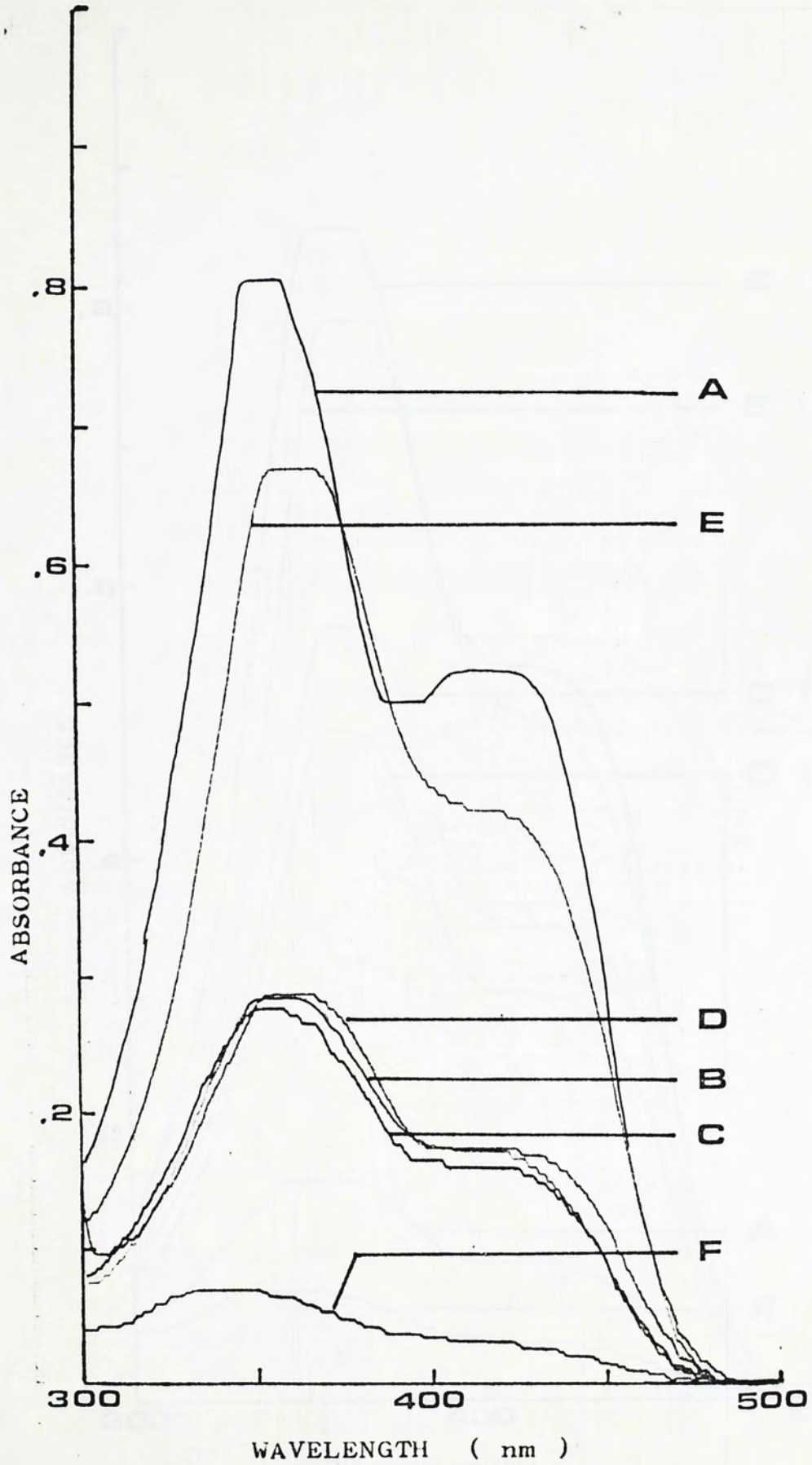


Figure 2.

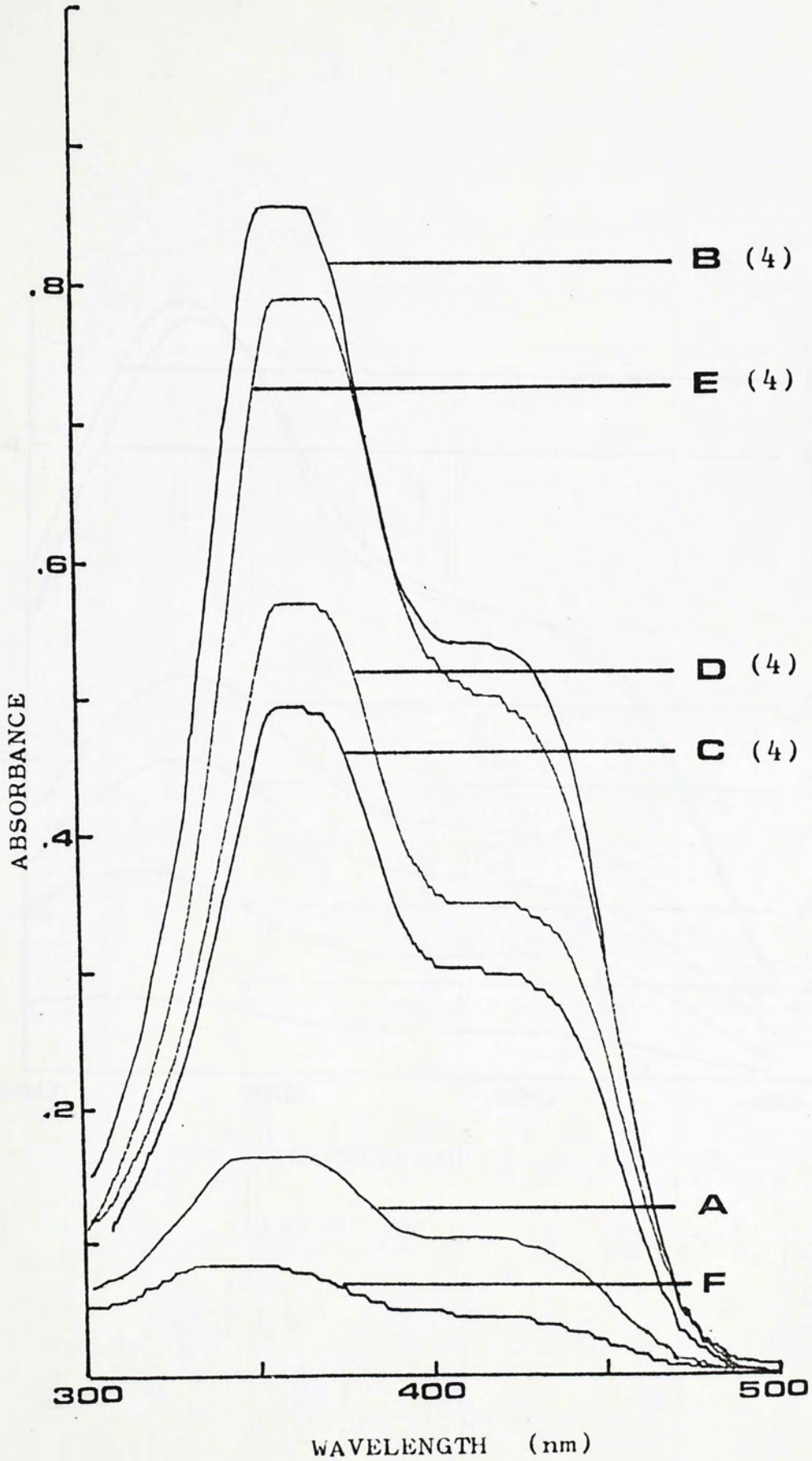
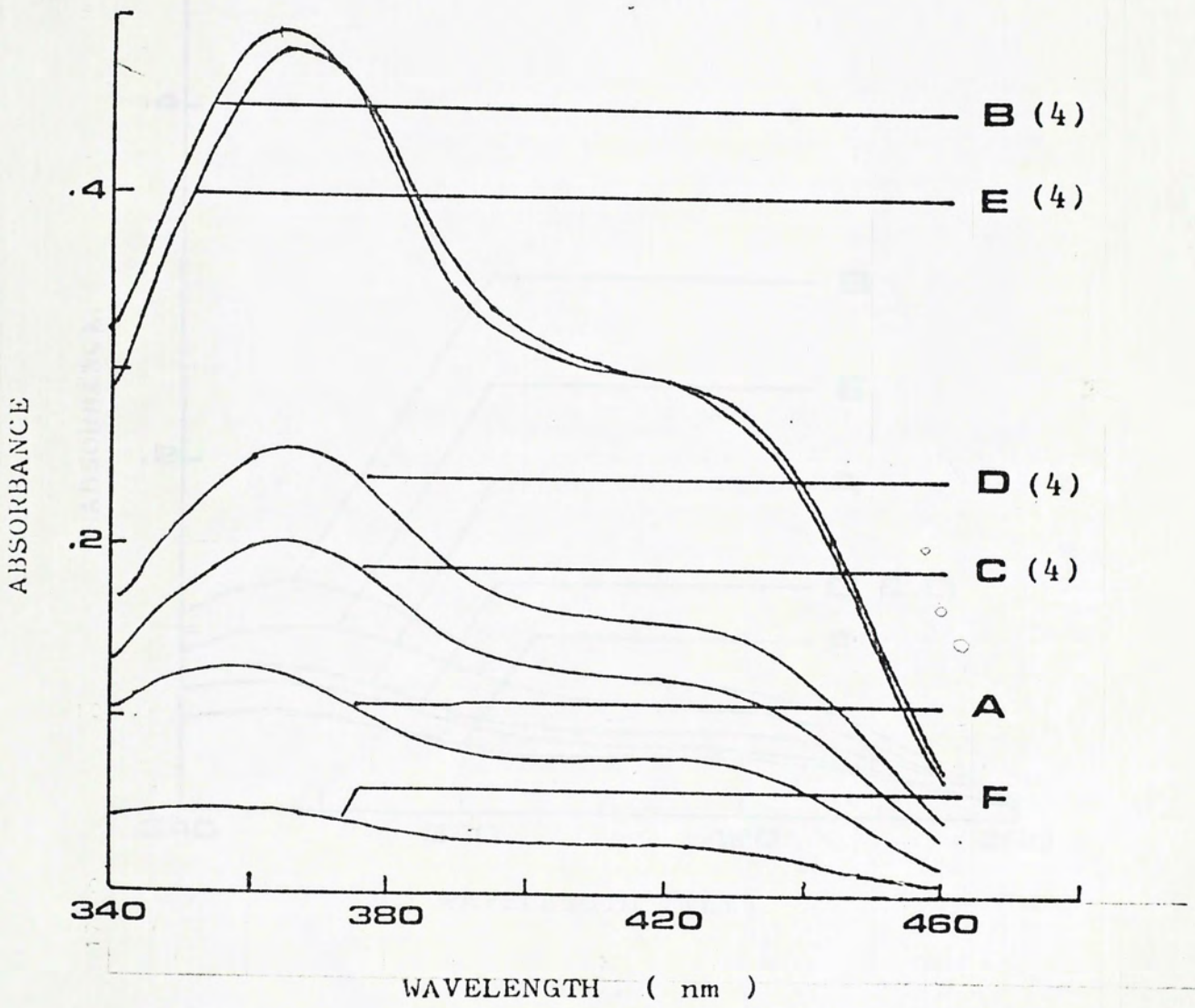


Figure 3.



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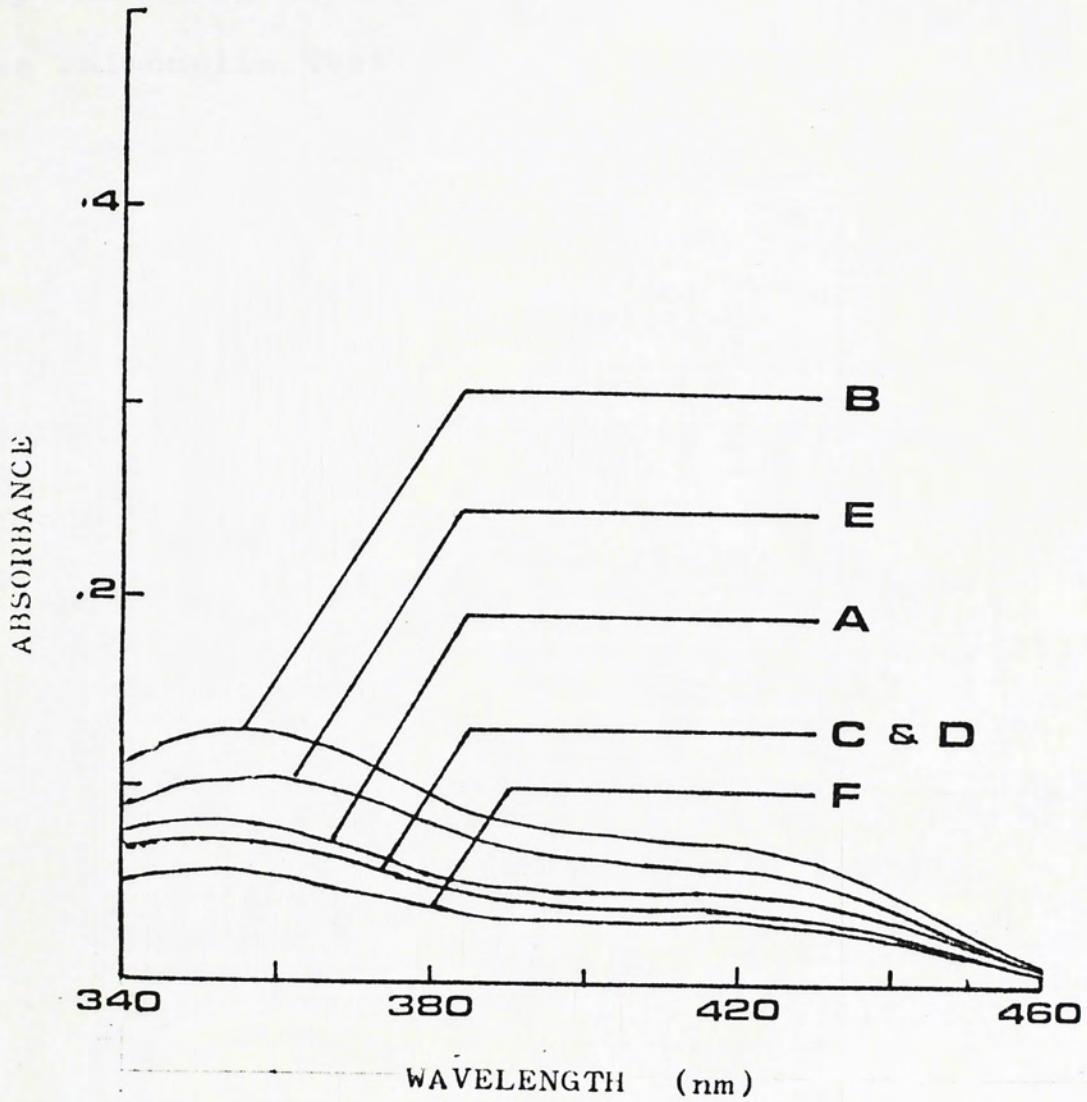


Figure 5.

APPENDIX II

Toxicity And Mutagenicity of Metal Salts Assayed by the Ames Salmonella Test

I. INTRODUCTION

1. Human Health Harzard Caused by Mutagens in the Environment

It has been suggested that carcinogenesis (Doll, 1977; Tomatis et al., 1978; Hiatt et al., 1977), genetic and developmental birth defects, heart desease (Benditt, 1977), aging (Burnet, 1974) and cataract formation (Jose, 1979) may have been caused by environmental mutagens. A damage in the DNA of the germ cell give rise to many genetic defects that may be carried on in future generations. A mutation in the DNA of the somatic cell, which alters the normal cellular processes as dictated by the correct DNA, in such a way as to upset the regulation of normal cell multiplication, may lead to carcinogenic outgrowth. At present, cancer and birth defects are among the most common diseases in the world population.

In the past, the problem of screening environmental mutagens was largely ignored. People get across to many chemicals for decades before these compounds were carefully tested for possible carcinogenicity or mutagenicity. This project is thus initiated to check the mutagenicity of chemicals, employing the Ames' test.

2. The Ames' Salmonella Mutagenicity Test

The Salmonella test was established by Ames for detecting chemical mutagens (Ames, 1971 & 1972; Ames et al.,1973a & b; McCann et al., 1975a). The test makes use of the potency of inducing reverse mutation in specialiyzed mutant Salmonella typhimurium (Ames et al. 1975). The test has gained wide acceptance because of its great efficiency in identifying carcinogens as mutagens (McCann et al., 1975a; McCann & Ames, 1976; Donahue et al.,1978). Of all common compounds tested, about 90 percent of the carcinogens were checked to be mutagenic by the Ames test. Because of its simplicity, inexpensiveness, less time consuming and high sensitivity in identifying mutagens, the Ames test has been routinely used as a substitute for the animal bioassay. In fact, when a rapid, inexpensive test is required, the Ames' test is the prefered one. However, it should be pointed out that the Ames' test reports the mutagenicity of the chemicals on the bacterial system, and there should be ground given to a correction when eukaryote is dealt with.

3. The Mutagenicity of Trace Metal Ions

Certain metal ions, such as Be, Cd, Co, Cr, Cu, Mn, Ni have been found to be toxic at higher dosage. Results from epidemiologic test and animal test have suggested that these metal ions are carcinogens. A brief review of these metal ions for their carcinogenic nature is tabulated (Table 1).

These elements being in their ionic forms are electrophilic and might react well with nucleic acid (Sharpiro, 1968). A correlation of the electronegativity and the carcinogenicity of the metal ion indicates that most carcinogens have electronegativity ranging from 1.2 to 1.9 (Table 2). It is suggested that these carcinogenic metals are capable of forming either covalent or coordination compounds with biochemically important legands. They include, among nucleic acid : guanine, adenine and cytosine; and the tertiary phosphate groups of the nucleic acids; among amino acid residues in protein : methionine, cysteine, tyrosine and histidine; and other nucleophilic centres of smaller molecular weight tissue components. While the importance of any of these reactions to carcinogenesis is unknown, the ability of the identified carcinogenic metal ions to exhibit strong electrophilicity *in vivo* does appear to be essential

Table 1 Carcinogenicity of Trace Metals

Element	Organism	Reference
Be	Human, beryllosis victims (cancer)	Stockinger, 1966
	Experimental animals (tumor)	Schepers et al., 1957
		Vorwald et al., 1966
	Rabbit	Tapp, 1966
Cd	Rats (tumor)	Haddow et al., 1964a
	Fowls (tumor)	Guthrie, 1964
Co	Human (thyroid gland tumor)	Weaver et al., 1956
Cr	Human, Cr mine workers (lung cancer)	Schepers, 1971
	Human (bronchial carcinomas)	Payne, 1960
Cu	Human, coppersmiths (cancer)	Agnese et al., 1959
	Rat (bone & lung tumor)	Stanton, 1967
Mn	Mice (lymphosarcomas)	Paola, 1964
	E. coli (mutation)	Demerec & Hanson, 1951
Ni	Human, Ni mine worker (lung cancer)	Doll, 1958
		Morgan, 1958

Table 2 Electronegativity and Carcinogenicity of Metals*

Electronegativity	Chemical Carcinogenicity		
	Carcinogen	Suspected	Inert
0.7			Cs
0.8			K, Rb
0.9			Na, Ba
1.0			Li, Ca, Sr
1.1			La
1.2		Y	Ha
1.3		Sc	
1.4	Zr		
1.5	Be, Cr	Al, Ti	
1.6	Zn	Ga	V
1.7	Cd		W, In
1.8	Co, Ni, Sn, Pb	Mn, Fe, Si	Mo, Tl, Ge
1.9		Cu	Tc,Re,Hg,Sb,Bi
2.0		As	
2.1			Te
2.2		Rh	Ru,Os,Ir,Pt
2.4	Se		Ag, Au

* Data taken from Luckey & Venugopal (1979)

(Miller & Miller, 1971).

In this investigation, an attempt is made to survey intensively and more systemetically the mutagenicity of metal ion of Ag, Be, Cd, Co, Cr, Cu, Mn and Ni with the Ames' Salmonella mutagenicity test.

II. MATERIALS AND METHODS

MATERIALS

1. Chemicals : Metal salt $AgNO_3$, $MnCl_2$, $Be(NO_3)_2 \cdot 4H_2O$, $NiCl_2 \cdot 6H_2O$, $CrCl_3 \cdot 6H_2O$, $CoCl_2 \cdot 6H_2O$, $CuCl_2 \cdot 2H_2O$ were purchased from Merck; CrO_3 from British Drug House; and $CdCl_2 \cdot 2.5H_2O$ from Hopkin Williams. All these metal salts used were of analytical grade. 9-amonoacridine was obtained from Sigma.

2. Bacterial Strains : Wild type Salmonella typhimurium LT_2 and its mutant strains TA1535, TA1537, TA1538, TA98 and TA100 were kindly provided by Prof. B.N. Ames. Table 3 described the genotypes of these mutant strains. The mutant strains contain mutation in the histidine operon and require histidine for growth. TA1535 and TA100 carry the hisG46 allele which can be reverted by base-pair substitutions; and TA1537, carrying the hisC3076 allele, as well as TA1538 and TA98, carrying the his D3052 allele, can be reverted by frame-shift mutations. In addition, they all lack the excision repair system (ΔuvrB mutation) and the lipo-polysaccharide barrier that coats the surface of the bacteria (rfa mutation). The strains TA98 and TA100 carry the plasmid R factor

Table 3 Genotype of Salmonella typhimurium Tester Strains

Histidine Mutation			Additional Mutation		
hisG46 ▽	hisC3076 △	hisD3052 △	LPS*	Repair	R factor ‡
TA1535	TA1537	TA1538	rfa	ΔuvrB	-
TA100	-	TA98	rfa	ΔuvrB	+R

* All strains possess a single deletion through the gal, chl, bio and uvrB genes.

‡ Inclusion of R factor confers resistance to ampicillin.

△ Revertable by frame-shift mutation.

▽ Revertable by base-pair substitution mutation.

pKM101 for ampicillin resistance which enable them to be more sensitive for mutagen detection.

3. Growth Medium

Nutrient Broth : 0.8% (w/v) Difco nutrient and 0.5% (w/v) NaCl in glass-distilled water was sterilized by autoclaving at 121°C for 25 minutes.

Nutrient Agar Plate : 1.5% (w/v) Difco Bacto-agar was added to nutrient broth and autoclaved. A 100 x 15 mm style plastic plate contains usually 25 ml of the nutrient agar.

Minimal Agar Plate : The agar medium, containing 1.5% Difco Bacto-agar and 2% D-glucose in Vogel-Bonner medium E (Vogel & Bonner, 1956), was sterilized by autoclaving (D-glucose was autoclaved separately). Approximately 25 ml of agar medium was poured into each sterile plastic plate (100 x 15 mm style). These plates were usually set overnight in a 45°C incubator before use to remove excess humidity.

Top Agar : Top agar containing 0.6% (w/v) Difco Bacto-agar and 0.5% (w/v) NaCl was autoclaved and stored in bittles in volume of 100 ml. Before use, the agar was melted by heating the bottle with its content in a

steam bath and stood at a 60°C water bath ready for use. A sterile solution of 0.5 mM L-histidine HCl and 0.5mM biotin was added to the molten agar with thorough mixing. To pour overlay, 0.1 ml of bacterial culture was mixed with 2 ml of the 60°C molten top agar and poured evenly onto the agar plate.

METHODS

1. Examination of the Tester Bacterial Strains

Checking out of the mutant strains, as described by Ames (Ames et al., 1975), is consist of the following steps :

A. Histidine Requirement

To a minimal plate supplemented with 0.1 ml of 0.5mM biotin either with or without 0.1 M L-histidine, a suspension of the tester strain was streaked on . Inoculated plates were incubated at 37°C overnight. Bacterial colonies formed were observed in which, the absence of bacterial colony in plates containing no histidine indicates that the tester strain has a straight requirement of histidine for growth.

B. The Deep Rough Character (rfa)

A sterile filter paper disc containing 10 µl of 1 mg/ml crystal violet was placed onto a nutrient agar plate containing 0.1 ml of the nutrient broth culture to be tested (about 10^8 bacteria) in a thin top agar overlay. After 16 hours incubation at 37°C, a clear ring inhibition zone around the disc indicates the presence of the rfa mutation in the strain.

C. The R Factor

A sterile filter paper disc containing 10 µl of 3 mg/ml ampicillin in o.o2 N NaOH was placed onto a nutrient agar plate with a thin overlay containing the tester strain culture. Again, after 16 hours incubation at 37°C, the absence of inhibition zone around the disc indicates the presence of ampicillin resistant R factor in the strain.

D. The uvrB Deletion

Nutrient agar plates containing 0.1 ml of the nutrient broth culture to be tested (about 10^8 bacteria) in a thin top agar overlay were regionally shadowed with glass microslides and were irradiated for 8 seconds with a UV lamp (15 w) fixed at a distance of 33 cm.

After overnight incubation at 37°C (about 16 hours), the UV-sensitive (ΔuvrB) strains appear only in the shadowed region where UV illumination was not transmitted.

2. Determination of Toxicity Range of the Metal Ions on Salmonella typhimurium LT_2

A 0.1 ml overnight tryptone broth culture (about 10^8 bacteria) of the parent Salmonella typhimurium LT_2 strain was mixed with warm top agar and poured as a thin overlay onto the nutrient plate. Sterile filter paper discs containing various concentrations of membrane (pore size 0.45 μm) filtered sterile aqueous solution of metal ion were placed on the plate. The metal ion concentrations tested are 10^{-6}M-10^{-1}M at 10-fold dilution intervals. After overnight incubation at 37°C (about 16 hours), a clear inhibition zone around the disc of known concentration indicates that concentration of the metal ion inhibits growth.

3. Mutagenicity Test for Metal Ions

A 0.1 ml overnight culture of the 5 mutant tester strains grown in nutrient broth was mixed with 2 ml soft top agar at 50°C and poured on the minimal agar plate. Sterile filter paper discs containing the metal

ion solution starting with low sublethal concentration up to the lowest level of the toxic concentration (usually 10^{-3}M - 10^{-1}M) were tested on plates containing each of the 5 mutant tester strain. After 48 hours incubation at 37°C, the plates were checked to see whether a ring of colonies was formed around the disc or not. The existance of colony ring indicates that the chemical tested is a positive mutagen.

In all experiments, a positive mutagenic control was included to confirm the reversion property of each mutant strain. A characteristic reversion property of the standard tester strains to some known mutagens are listed in Table 4. Only 9-aminoacridine, used at 10 µl of 1 mg/ml in ethanol, was used for diagnosting the reversion property of TA1537.

Table 4 Recommended Strain-specific Potisive Control Compounds*

Strain	Compound
TA1535	Ethyl methanesulfonate or sodium azide
TA1537	9-Aminoacridine
TA1538, TA98	4-Nitro-o-phenylenediamine, 2-nitrofluorene or hycanthone methanesulfonate
TA100	Sodium azide, methyl methanesulfonate or nitrofurantoin

* Informations obtained from Serres & Shelby (1979).

II. RESULTS

1. The Genotype of the Tester Strains

All 5 mutant strains of Salmonella typhimurium LT_2 were checked to be histidine autotrophic, having shown that no colony formation was reported on minimal plate without histidine. They all showed clear inhibition ring to crystal violet (Fig. 1), indicating the existance of deep rough character by the rfa mutation. They were all sensitive to UV irradiation, as in Fig. 2. The additional R factor of TA98 and TA100 was present, as indicated in Fig. 3, ampicillin induced clear inhibition zone to the three tester strains TA1535, TA1537 and TA1538, but no inhibition in the two strains TA98 and TA100.

2. Metal Ion Toxicity on Salmonella typhimurium LT_2

The metal salts tested showed inhibition to growth of the parent Salmonella typhimurium LT_2 strain at higher dosage range, 10^{-3}M to 10^{-1}M, as indicated by a clear inhibition ring (Fig. 4). The width of the inhibition ring varies among the metal salts, probably due to the variation in toxicity potency as well as the diffusion rate of the metal salts. It was also noticed that the appearance of the inhibition rings also differed

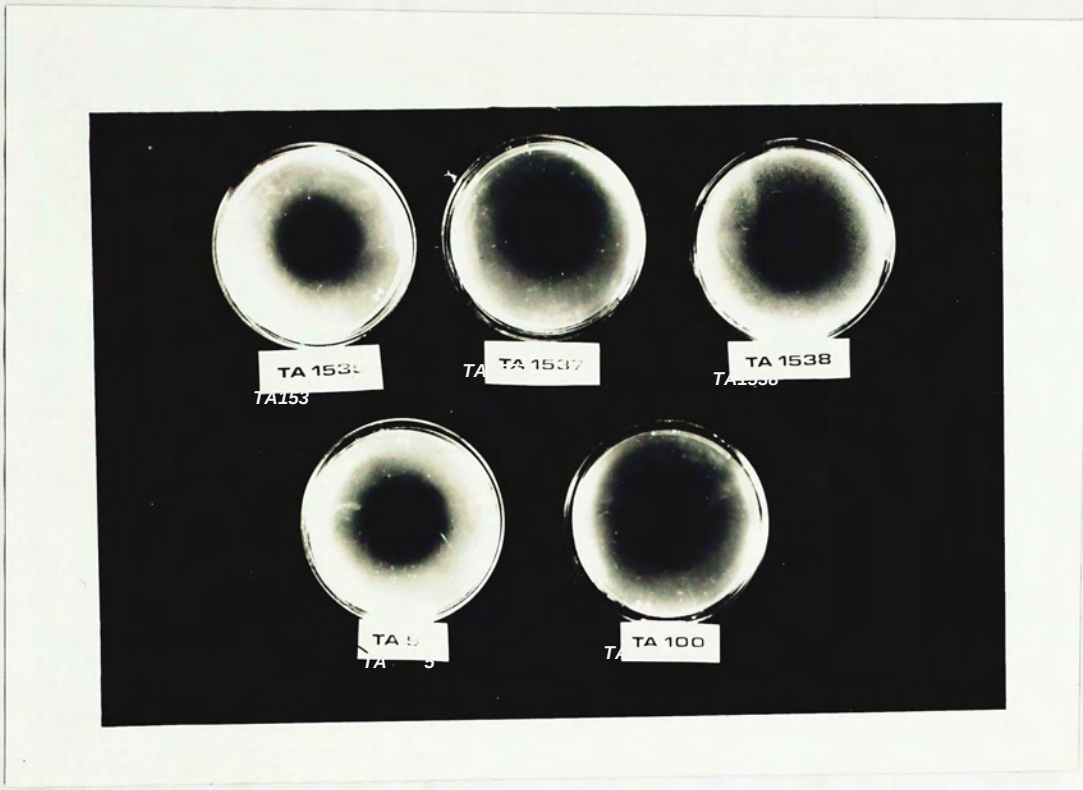


Figure 1 Inhibition of Crystal Violet to the Tester Strains

The nutrient plates were poured with strains as marked. The filter paper disc at the centre of each plate contained 10 µl 1 mg/ml of crystal violet. A dark ring around the disc is the inhibition ring.

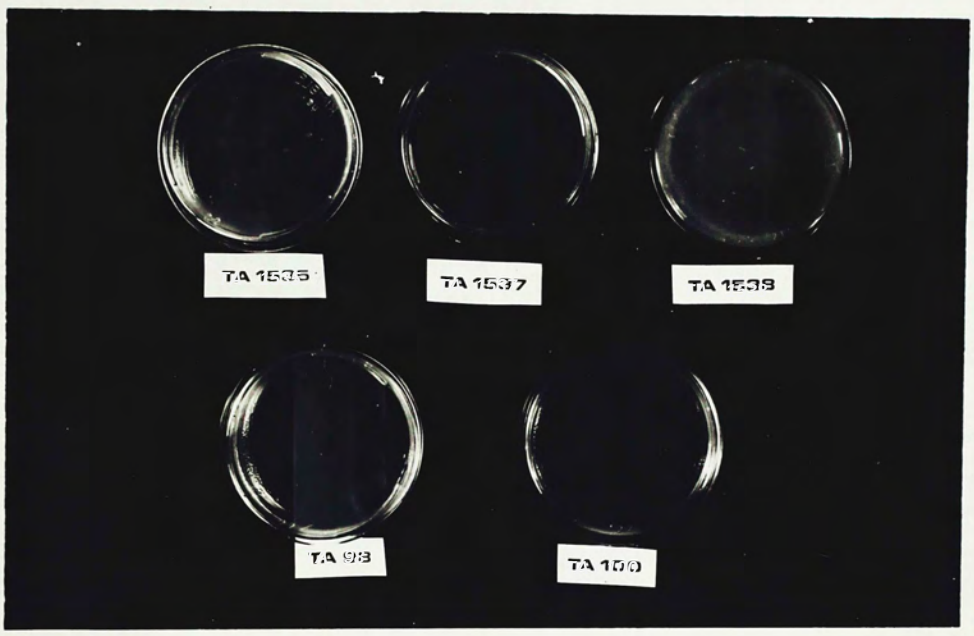


Figure 2 Effect of UV Irradiation on the Tester Strains

The nutrient plates were poured with the strains as marked. the darker area indicates the bacterial growth inhibition area not shielded from UV irradiation.

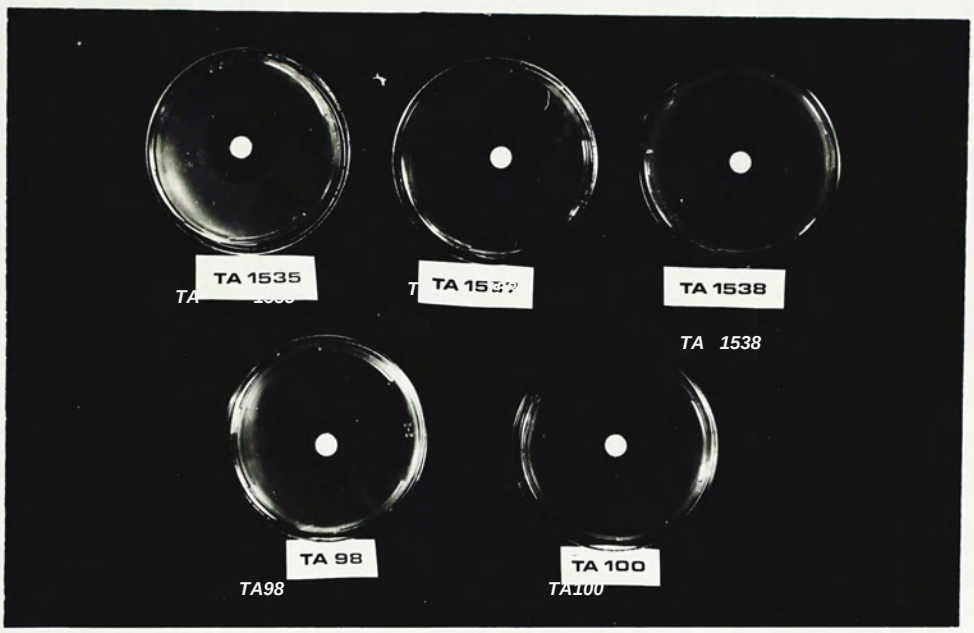
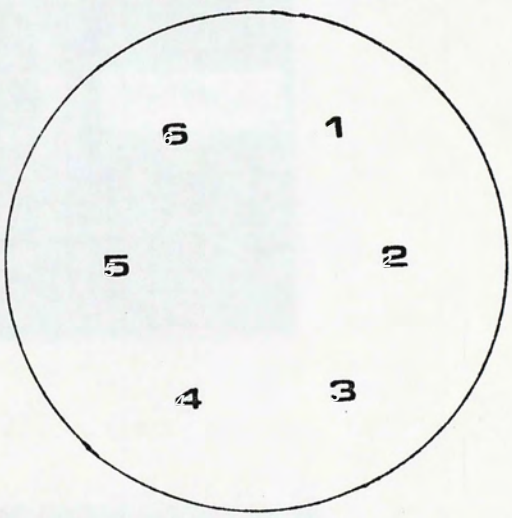


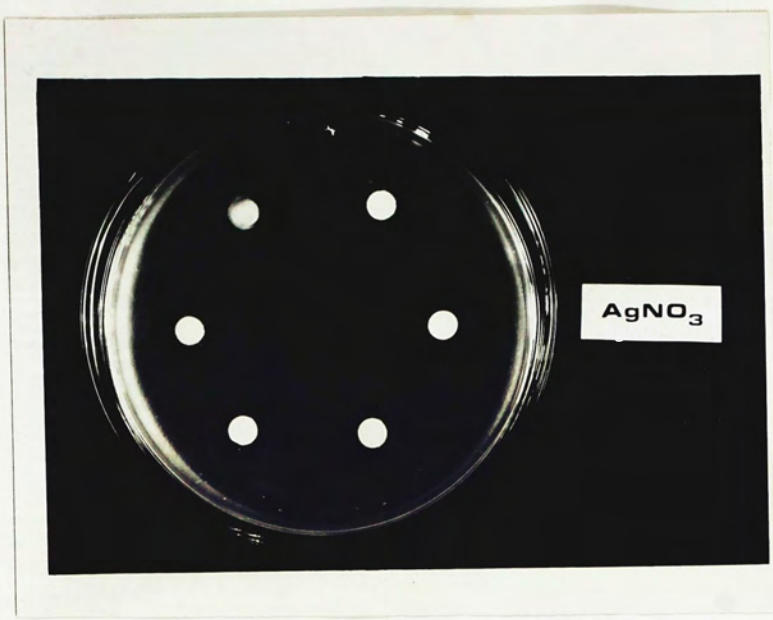
Figure 3 Effect of Ampicillin on the Tester Strains

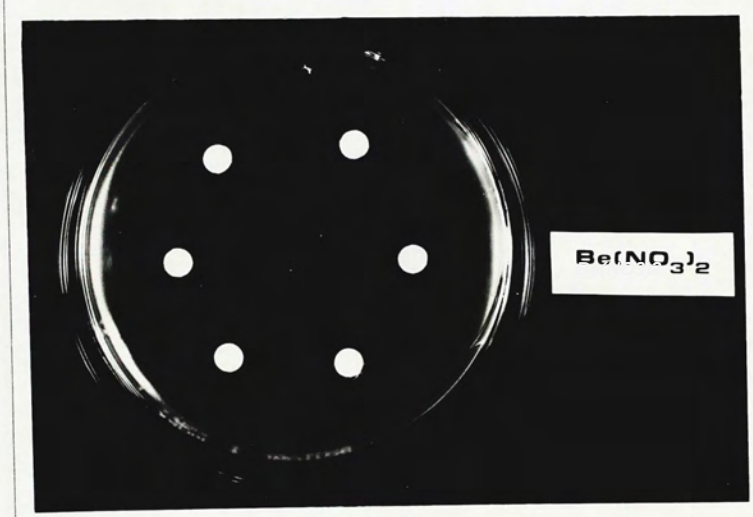
The corresponding tester strains (as marked) was incorporated on nutrient plate. The disc contained 10 µl of 8 mg/ml ampicillin in 0.02 N NaOH. A darker area indicates a clear inhibition zone. Note the absence of the inhibition zone in the two strains TA98 and TA100.

Figure 4 Toxicity of Metal Salts on Salmonella typhimurium LT_2 Strain

Ten ul of metal salt solution were added on each disc diameter 5 mm. Concentrations of metal salts in each disc reading from position 1 to 6 in the sample diagram were 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2} and 10^{-1} M respectively.



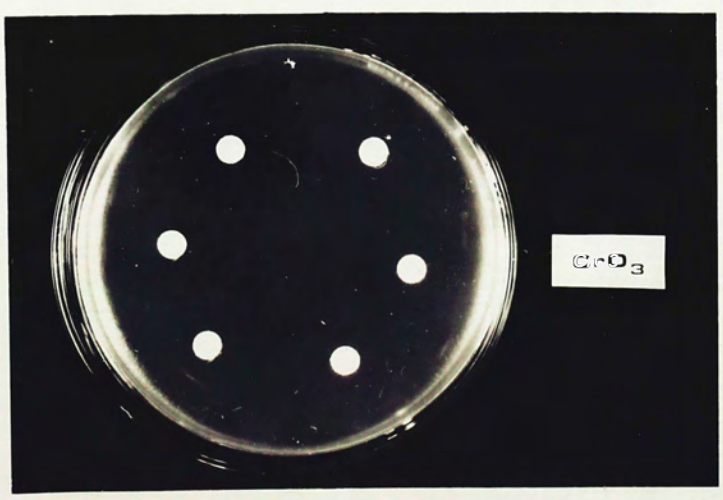

$AgNO_3$

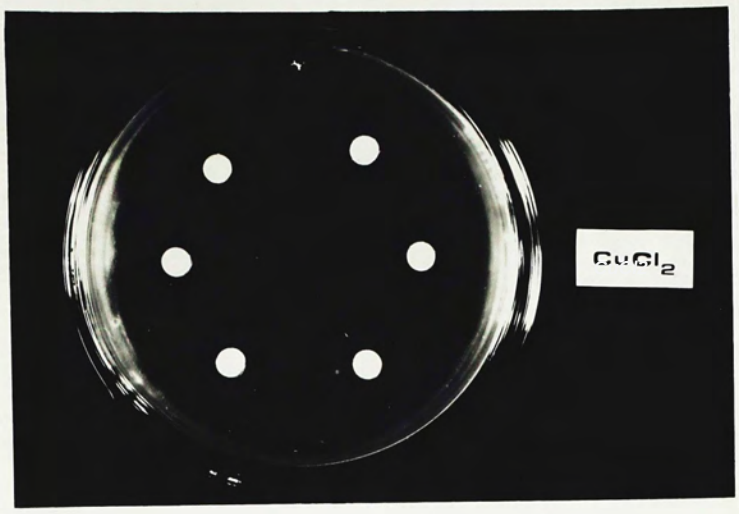

$Be(NO_3)_2$


$CdCl_2$

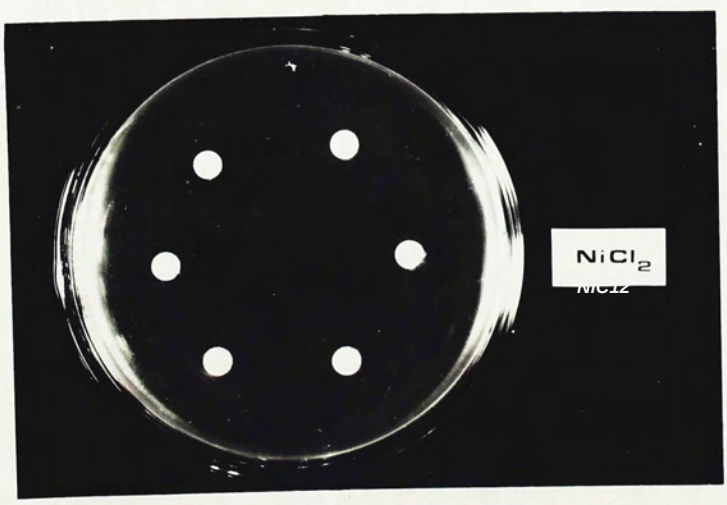

$CoCl_2$


$CrCl_3$


CrO_3


$CuCl_2$


$MnCl_2$


$NiCl_2$

among the metal salts. Some inhibition rings showed clear boundary, such as that induced by the metal salts of Be, Co, Cr(III), Cu and Ni. While others had a more diffused boundary, such as that induced by the metal salts of Ag, Cd and Cr(VI). At careful examination, the inhibition ring induced by the salt of Cr(VI) was actually very distinctive in appearance, with microcolonies distributed in a concentric manner around the disc, within the region of the inhibition zone (Fig. 5). While the turbid ring induced by metal salts of Mn and Cd were further verified to be artifacts not related to bacterial growth, probably due to precipitation of the metal salts caused by the complex formation with the components in the agar plate, since similar turbid ring also occured at plates without inoculation of bacteria.

3. Mutagenicity of Metal Salts Assayed by the Ames Salmonella Test

9-aminoacridine, the recommended positive control for TA1537 strain, induces a distinct ring of reverse colonies around the disc, as shown in Fig. 6.

Metal salts selected for the study were simultaneously screened in the mutagenicity test, at concentration ranging usually from 10^{-3}M to 10^{-1}M. All expe-

Figure 5 Distinct Appearance of CrO_3 Inhibition Ring on Salmonella typhimurium LT_2

Panel A is the inhibition ring induced by CrO_3 and panel B is that by $NiCl_2$. The filter paper discs contained 10 µl of 10^{-1}M (right hand side position) and 10^{-2}M (left hand side position) metal salt solutions.

A

B

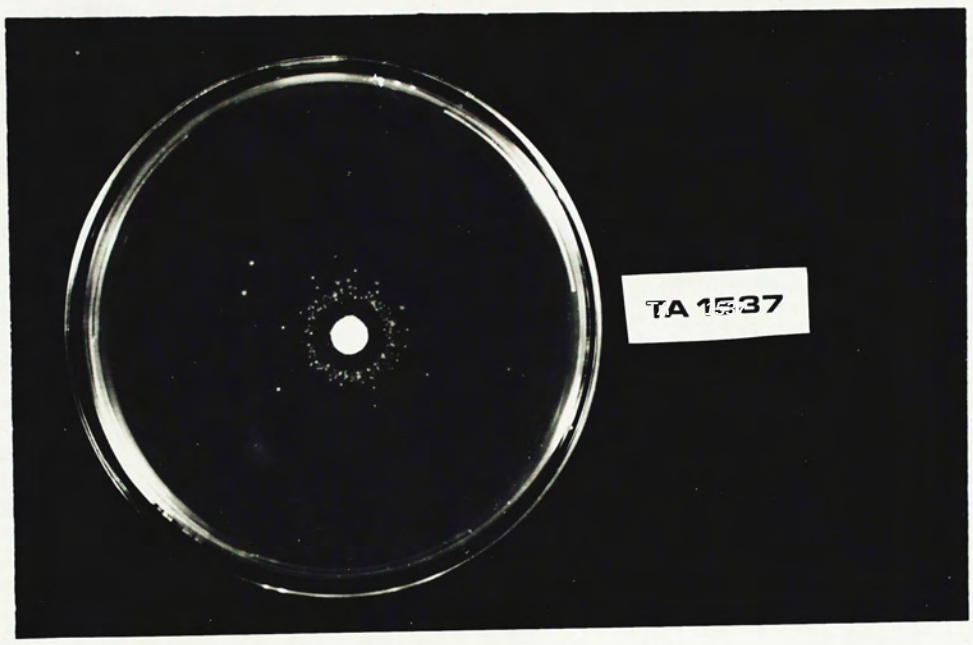


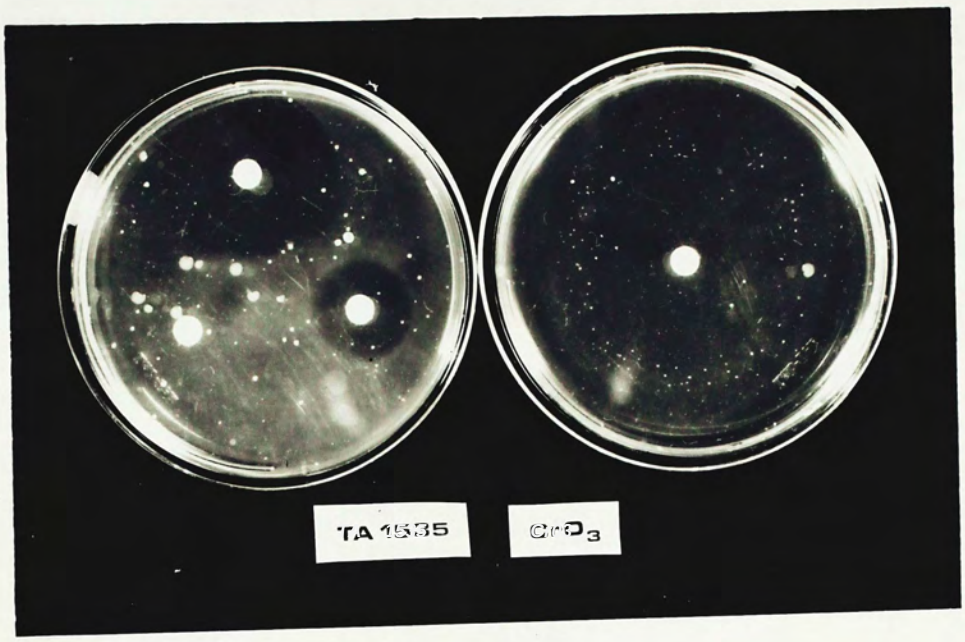
Figure 6 Mutagenicity of the Mutagen 9-Aminoacridine on the Tester Strain TA1537

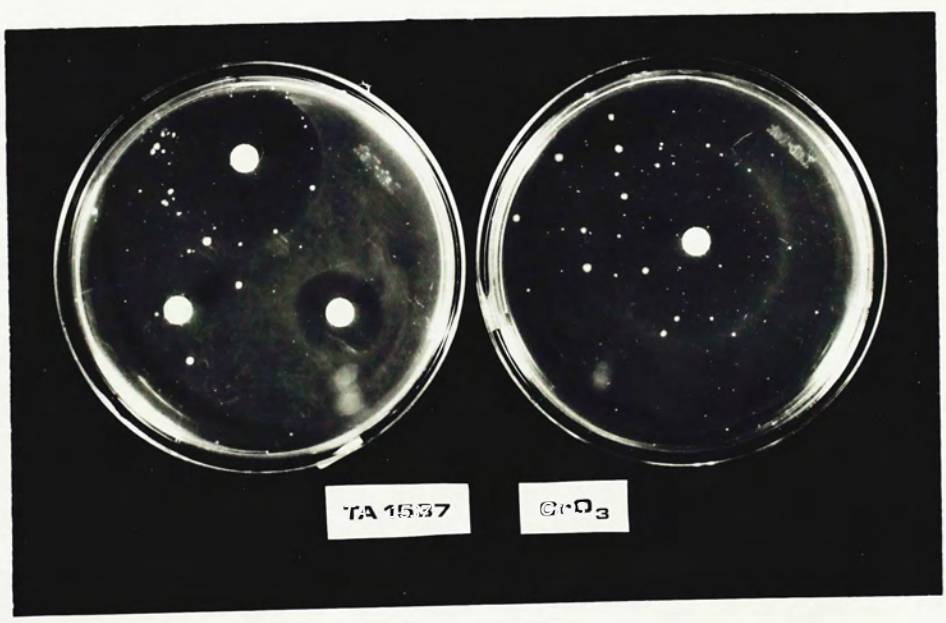
Reversion induced by 9-aminoacridine. Mutant strain employed was TA1537.

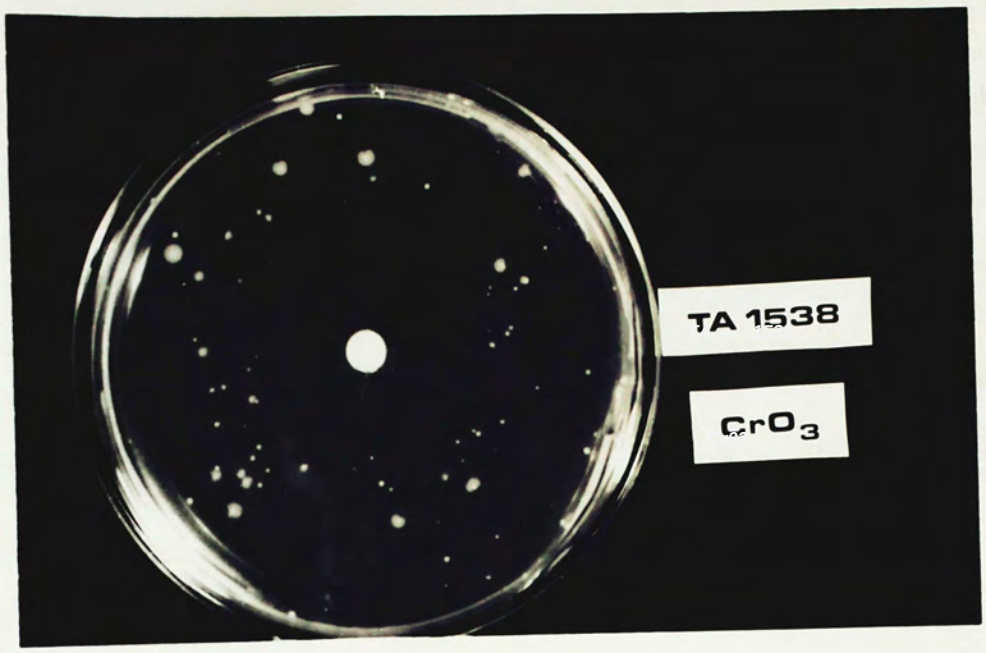
riments were tested at least three times to confirm the observations. The results indicated that all metal salts tested showed negative mutagenicity except CrO_3, which induced rings of reverse colonies in all five tester strains (Fig. 7).

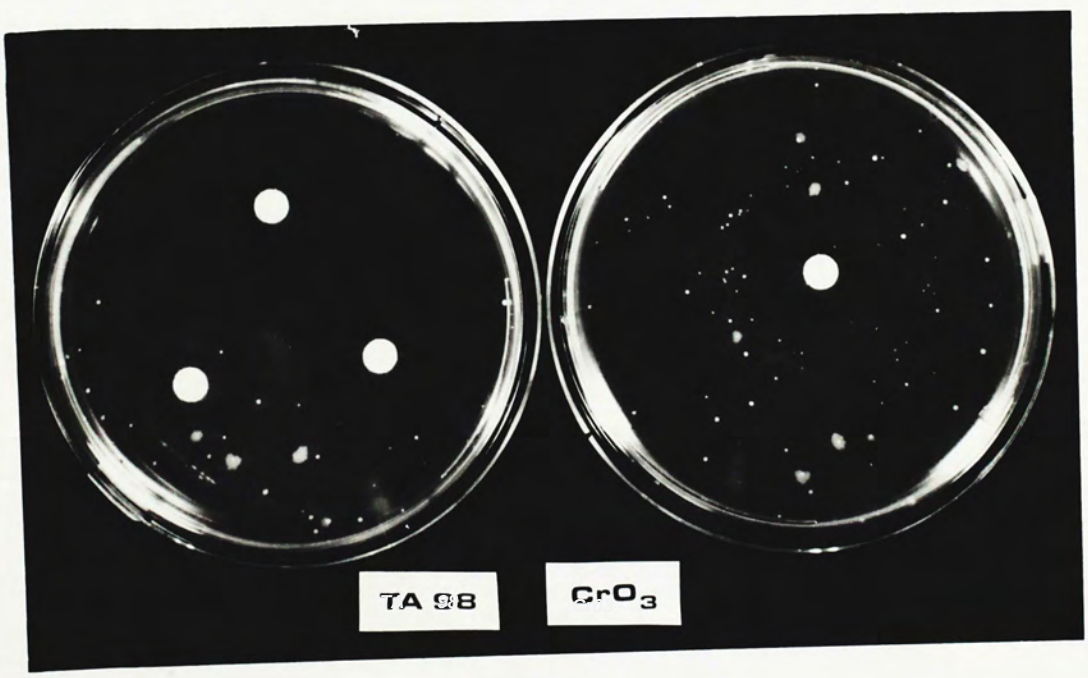
Figure 7 Mutagenicity of the Metal Salt CrO_3 on the Tester Strains

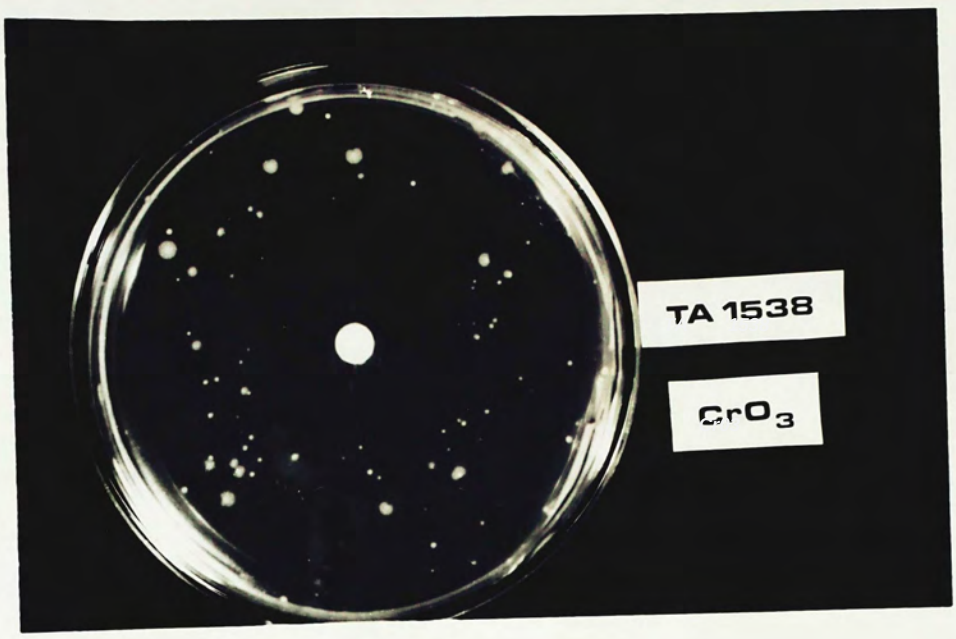
Reversion colonies induced by CrO_3 on the five tester strains TA1535, TA1537, TA1538, TA98 and TA100. Plates with 3 discs contained 10^{-3}, 10^{-2} and 10^{-1}M of the metal salt solution (starting to read from the uppermost position clockwisely). Plates with single disc contained 10^{-1}M of CrO_3 for strains TA1535, TA1537 and TA1538; and 10^{-2}M for strains TA98 and TA100.

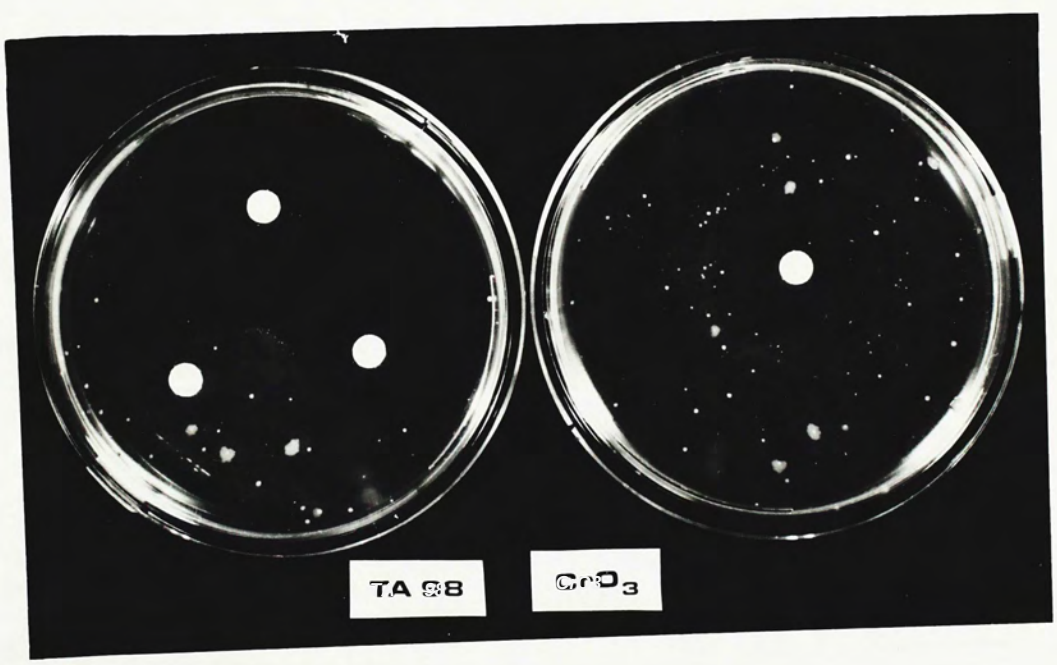

TA 1535
CrO_3


TA 1537
CrO_3


TA 1538
CrO_3


TA 98
CrO_3


TA 1538
CrO_3


TA 98
CrO_3


TA 100
CrO_3

VI. DISCUSSIONS

The metal salts selected in the study showed inhibitory effect to Salmonella typhimurium LT_2 strain at higher concentration range, about 10^{-2} M. It was suggested that the toxicity of the metal ions are related to their physicochemical properties such as electrochemical nature, oxidation state, solubility and stability in the biological system (Luckey & Venugopal, 1979). Their toxic effect in biological systems appears to have resulted from their interactions with proteins or nucleic acids thus interfering with the normal physiological function of the system.

Among all metal salts assayed by the Ames test, only CrO_3 showed positive mutagenicity in all five mutant tester strains. Although the hexavalent chromium ion also has been shown to be mutagenic in the Ames test and other bacterial testing systems (Petrilli & Flora, 1977; Nestmann et al., 1979), it was reported that the mutant strain TA1535 was much less sensitive or undetectable to this metal ion at the oxidation state of six. However, in this project, hexavalent chromium ion exhibited significant mutagenicity in tester strain TA1535. This finding removes doubt in previous report on their nega-

tive result with TA1535 and because this tester strain represents a different mode of mutation mechanism, may add new information to our understanding of the hexavalent chromium ion mutagenicity. According to Ames et al., this tester strain TA1535 is a hisG46, missence mutation (Hartman et al., 1971), a reversion in this mutant strain indicates that the mutation mode is a base-pair substitution type (Ames, 1971 & 1972), whereas a reversion in either strain TA1537, a hisC3076 mutation (Oeschger & Hantman, 1970) or strain TA1538, a hisD3052 mutation (Hartman et al., 1971), indicates a frameshift mutation. Based on the results obtained here, that the hexavalent chromium ion is capable of reverting both types of tester strains, it is possible that the ion may cause mutation both in frameshift mutation and base-pair substitution. This is in agreement with the conclusion drawn by Tamaro et al.,(1975) and Petrilli et al. (1977). An alternation is that the metal ion interferes with the enzyme systems needed for accurate DNA replication.

Strains TA98 and TA100, derived from TA1538 and TA1535 respectively, by a tranfer of a plasmid R factor which enhances the error-prone recombinational repair potency, can dictate mutagens that work through this

repair system by giving an increase of reversion rate than their corresponding parent strains without the plasmid transfered. Therefore, a slight but significant enhancement in the reversion found in TA100 than its parent TA1535 by CrO_3, suggests that the metal mutagenicity in the base-pair substitution type may involve the error-prone recombinational repair system.

As in most reports, the mutant Salmonella typhimurium strains were employed instead of the wild type LT_2 strain in the mutagenicity assay. A finding that a distinctive hexavalent chromium induced microcolonies within the inhibition ring of the parent Salmonella typhimurium LT_2, might suggest an alternative simple way to test toxicity and mutagenicity with the parent LT_2 that is not reported previously.

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